

Synthetic and Biosynthetic studies on the Polyketide  
Metabolites LL-D253 $\alpha$  and averufin

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A thesis presented for the degree of  
Doctor of Philosophy

University of Edinburgh

1987



## DEDICATION

To my mother, and to the memory of my father.

## DECLARATION

I declare that this thesis is my own composition, the work of which has been carried out by myself, unless otherwise stated, and that it has not been previously submitted towards a higher degree or qualification. The research was undertaken in the Chemistry Department of the University of Edinburgh, under the supervision of Dr. Thomas J. Simpson, after the 1st. October 1981, the date of my admission as a postgraduate student.

## ACKNOWLEDGEMENTS

I would like to express my gratitude to my research supervisor, Dr. Thomas J. Simpson, for suggesting the research topics undertaken, and for his encouragement, enthusiasm and patience throughout the work described. I would also like to thank various technical staff of the Chemistry Department, University of Edinburgh, namely John Grunbaum and Elaine McDougall for microanalyses, Laurence H. Bell, John R.A. Miller, Dr. David Reed and Graeme Stevenson for n.m.r. spectra, and Elizabeth Stevenson for mass spectra. Particular thanks are due to Suzan Kasperek for advice and considerable practical assistance with all microbiological aspects of the work described. I would also like to thank numerous laboratory friends and colleagues, particularly Dr. C. Rupert McIntyre, for helpful discussion and advice.

The award of a S.E.R.C. studentship is gratefully acknowledged.



## ABSTRACT

In Chapter 1 a brief outline of the range of naturally-occurring oxygen heterocycles is presented. This is followed by a comprehensive review of non-flavanoid naturally-occurring chromanones, including reference to any important biosynthetic studies. This is followed by a review of the synthesis of such compounds, concentrating on more recent developments.

Chapter 2 concerns the biosynthesis of the chromanone LL-D253 $\alpha$ , a metabolite of *P. pigmentivora*. A review of previous studies is presented, followed by a brief account of the existence of the phenonium ion, thought to be an intermediate in the biosynthesis of LL-D253 $\alpha$ . The incorporation of sodium [2- $^2\text{H}_3$ ,  $^{13}\text{C}$ ]-acetate, and of diethyl [2- $^{13}\text{C}$ ]-malonate, into LL-D253 $\alpha$  give an insight into the mechanism of its formation. Chemical manipulation of deuterium labelled LL-D253 $\alpha$  derivatives has supported the existence of a "phenonium" intermediate. Chemical synthesis of some postulated precursors to LL-D253 $\alpha$  was undertaken.

Chapter 3 concerns the biosynthesis of averufin, known to be a precursor to aflatoxin B<sub>1</sub>. An overview of aflatoxin biosynthesis is presented, and includes a detailed review of relevant studies reported since 1980. The incorporation of [2- $^2\text{H}_2$ ]-hexanoic acid, and of diethyl [2- $^{13}\text{C}$ ]-malonate, into averufin is reported. These results confirm that averufin is a decaketide, but during its biosynthesis it is able to exchange with endogenous hexanoate. Attempted chemical manipulation of averufin, and incorporation of [7- $^2\text{H}_2$ ]-averufin into aflatoxin B<sub>1</sub> is discussed.

## LIST OF ABBREVIATIONS

Ac	acetate
Bz	benzyl
Et	ethyl
LDA	lithium diisopropylamide
Me	methyl
PCC	pyridinium chlorochromate
Ph	phenyl
p-TSA	para-toluenesulphonic acid
Ts	para-toluenesulphonyl
THF	tetrahydrofuran

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## Synthesis and Biosynthesis of LL-D253 $\alpha$ and its derivatives

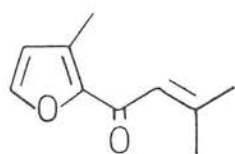
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# The Biosynthesis of Aflatoxin B<sub>1</sub>

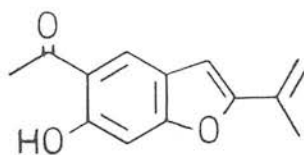
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CHAPTER 1

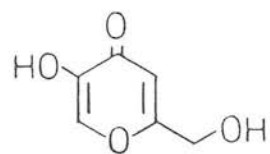
**NATURALLY-OCCURRING CHROMANONES  
AND THEIR SYNTHESIS**



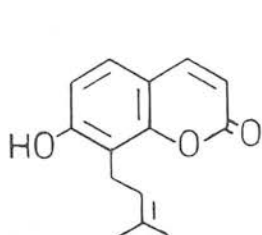
(1)  
naginata ketone  
*Perilla frutescens*<sup>4</sup>



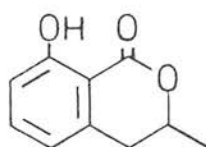
(2)  
euparin  
*Eupatorium purpureum*<sup>5</sup>



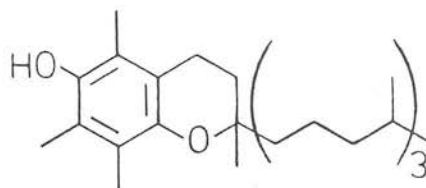
(3)  
kojic acid  
*Aspergillus* species<sup>6</sup>



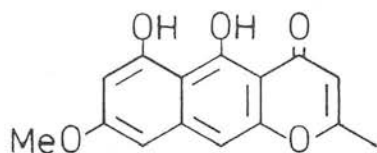
(4)  
osthenol  
*Angelica archangelica*<sup>7</sup>



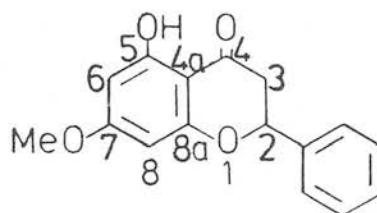
(5)  
mellein  
*Aspergillus melleus*<sup>8</sup>



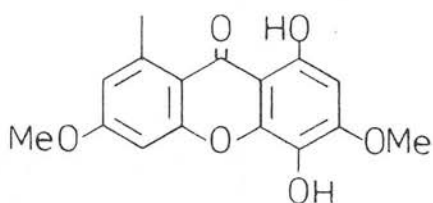
(6)  
 $\alpha$ -tocopherol  
Vegetable oils



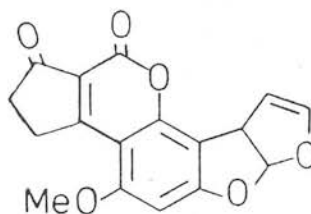
(7)  
rubrofusarin  
*Fusarium culmorum*<sup>9</sup>



(8)  
naringenin  
*Acacia* species<sup>10</sup>



(9)  
lichexanthone  
*Parmelia formosana*<sup>11</sup>



(10)  
aflatoxin B<sub>1</sub>  
*Aspergillus flavus*<sup>12</sup>

Figure 1: Naturally-Occurring Oxygen Ring Compounds.

## 1.1. Introduction

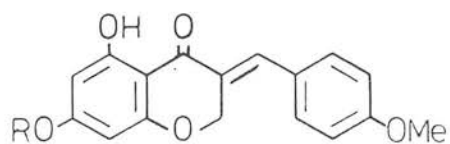
There are many types of oxygen-heterocycles occurring in nature<sup>1,2,3</sup>; representative examples of the more common types are shown in Figure 1. One extensively-studied group of compounds are those based on the [4H]-1-benzopyran skeleton. These include the chromans, 2H-chromenes, 4H-chromenes, 3-chromanones, 4-chromanones and chromones. For the latter three groups, the "4H-1-benzopyranone" nomenclature has been adopted by Chemical Abstracts. However the use of the terms "chromanone" and "chromone" persist in the literature due to their simplicity and convenience. This convention will be followed in the remainder of this text. Also, "chromanone" should be taken to mean "4-chromanone", unless otherwise stated. The numbering scheme used in the rest of this text is shown for structure (8) in Figure 1.

Of the naturally-occurring examples of the types of compound listed above, the most studied are the chromones. By far the most common examples are those containing a phenyl or aryl (particularly hydroxy or polyhydroxyphenyl) substituent at the 2-position. These are known collectively as flavones, and are produced by many plant species. Another important group are the isoflavones, which are 3-(phenyl or aryl) substituted chromones, and are known to be derived *in vivo* from flavones. Flavones and isoflavones have been extensively reviewed<sup>13</sup>. In contrast, chromones containing substituents other than phenyl or aryl occur rarely.

Chromanones, or 2,3-dihydrochromones, are less common in nature than chromones. Again they are produced mainly by plant species, and the phenyl or aryl are the most common substituents at the 2- or 3- positions. These are termed flavanones and isoflavanones respectively. These too have been extensively reviewed<sup>13</sup>. However, there are few examples of naturally-occurring

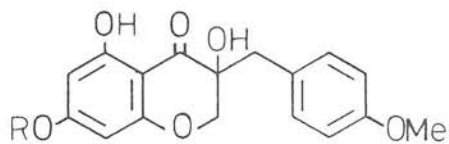
chromanones containing substituents other than phenyl or aryl at the 2-position.





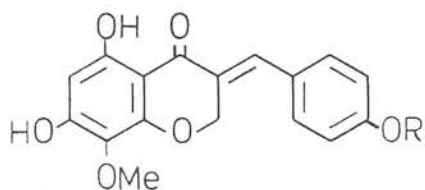
(11) R=H

(21) R=Me



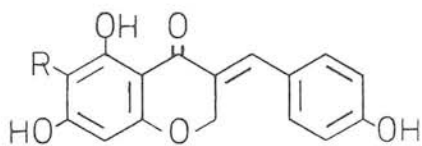
(12) R=H

(22) R=Me



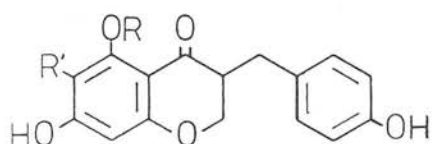
(13) R=Me

(16) R=H



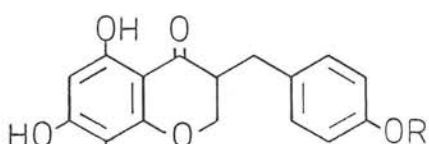
(14) R=OMe

(19) R=H



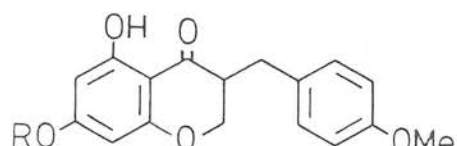
(15) R=H, R'=OMe

(20) R=Me, R'=H



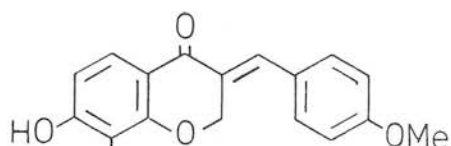
(17) R=H

(18) R=Me



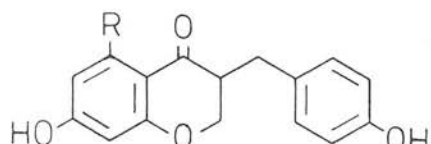
(23) R=H

(24) R=Me



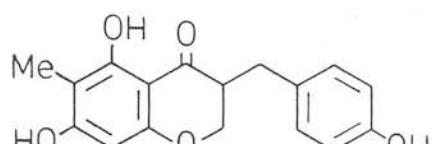
(25) R=H

(26) R=OMe



(27) R=H

(28) R=OH



(29)

## 1.2. Naturally Occurring Chromanones

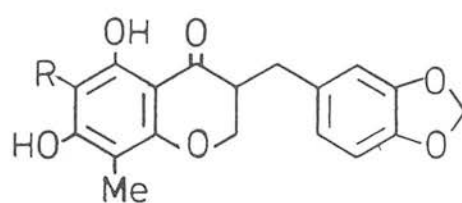
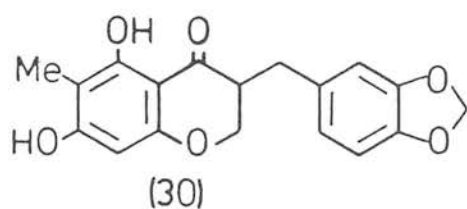
The scope of this review includes all naturally-occurring 4-chromanones which do not contain an aryl substituent at the 2- or 3-positions. A review of similar scope has been published recently<sup>14</sup>.

### 1.2.1. Homoisoflavanoids

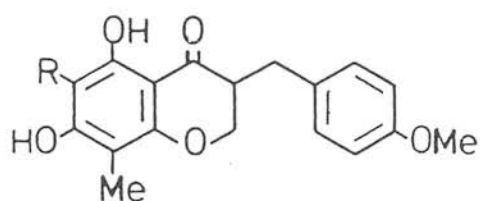
A group of metabolites known as homoisoflavanoids are produced by certain *Liliaceae* species. Eucomin (11) and eucomol (12) were first isolated from *Eucomis bicolor* in 1967 by Tamm's group<sup>15</sup>. In 1970 three further metabolites, 4'-O-methylpunctatin (13), autumnalin (eucomnalin) (14) and its 3,9-dihydro derivative (15) were isolated from *E. autumnalis*<sup>16</sup>. Punctatin (16), its dihydro- and 4'-O-methyldihydro-derivatives, (17) and (18), and 4'-demethyleucomin (19) and its saturated 5-methyl derivative (20) were isolated from *E. punctata* along with (11)<sup>17</sup>.

Further work by Tamm led to the isolation of 7-O-methyleucomin (21), (-)-7-O-methyleucomol (22) and 3,9-dihydroeucomin (23) and its 7-O-methyl derivative (24) from *E. bicolor* bulbs. He also demonstrated that the "E-" geometrical isomer (as written) of eucomin is an artifact, and that the "Z-" isomer is the genuine metabolite<sup>18</sup>. The absolute configuration of eucomol (12) was determined as (3S) in 1977<sup>19</sup>. At the time of writing, the last publication by Tamm concerning *Eucomis* metabolites reports the isolation of 5,7-dihydroxy-8-methoxy- 4-chromanone from *E. comosa*. This is the first reported naturally-occurring chromanone unsubstituted in the heterocyclic ring<sup>20</sup>. A few such *chromones* exist, and they may be derived from flavanoids *in vivo* or during isolation<sup>21</sup>.

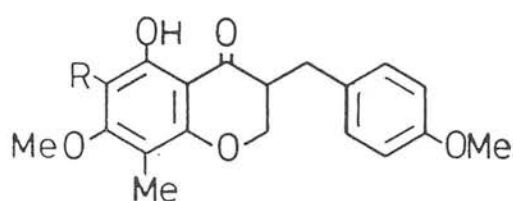
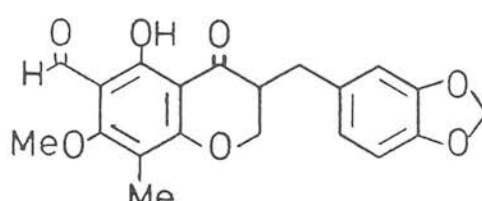
Recently further examples of homoisoflavones from other species have been reported. Bonducellin(25) was isolated from *Caesalpinia bonducella*<sup>22</sup>, its



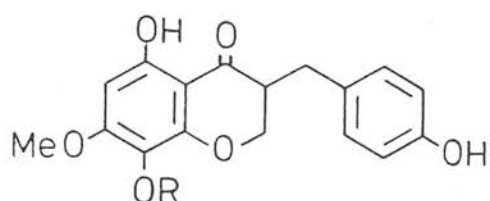
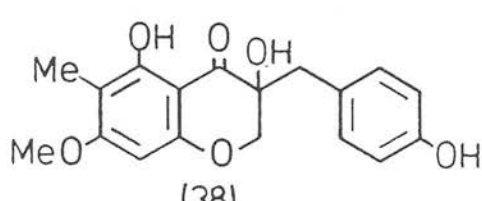
(33) R=CHO



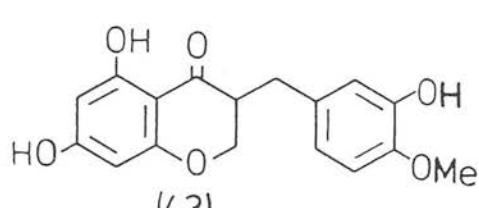
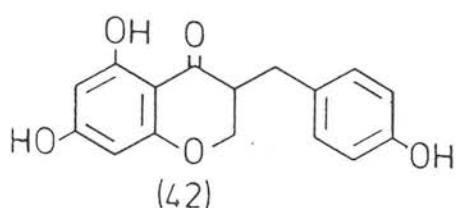
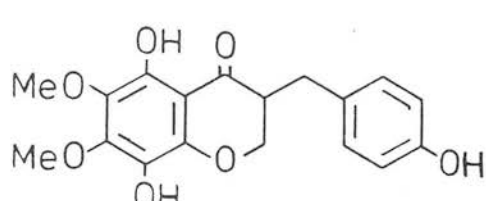
(34) R=CHO

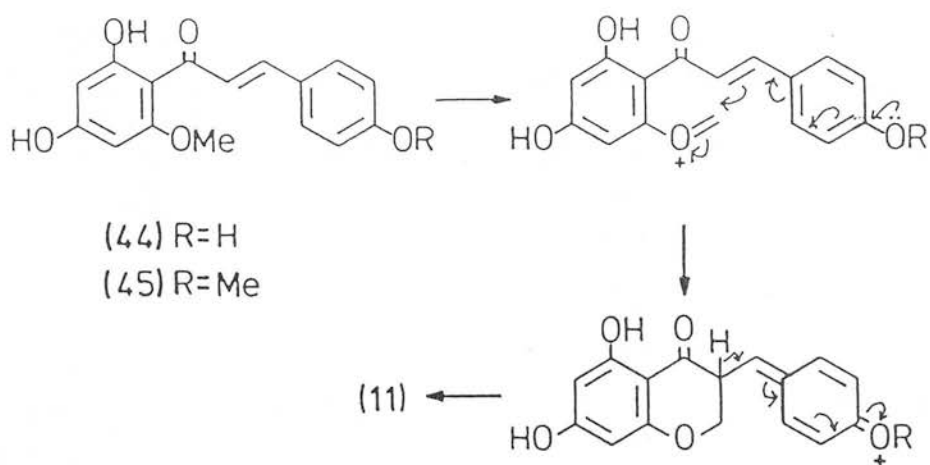


(37) R=CHO

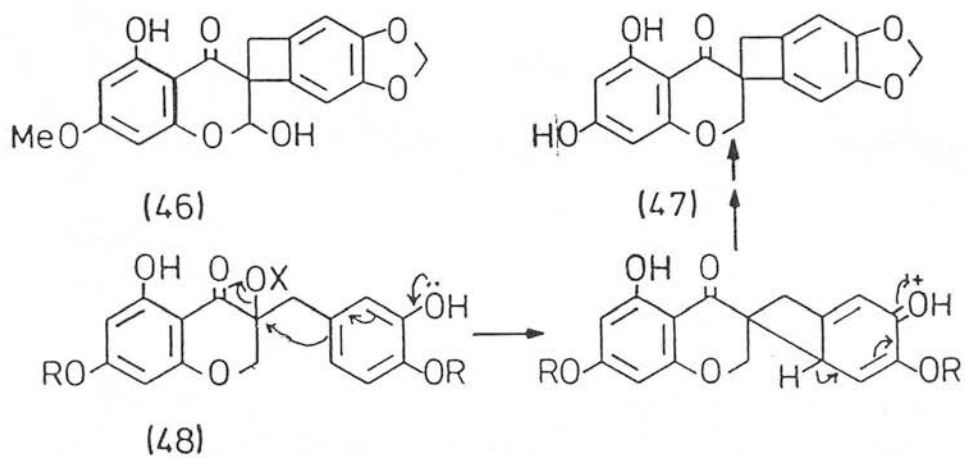


(40) R=Me

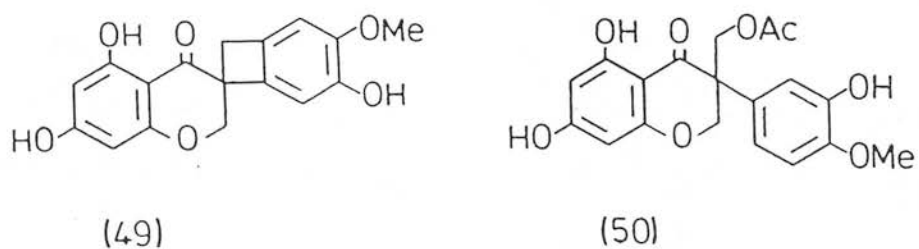




Scheme 1



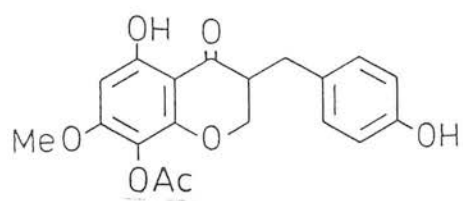
Scheme 2



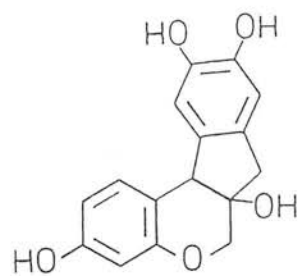
8-methoxy analogue (26) from *C. pulcherrima*<sup>23</sup>, and (27), (28) and (29) from *Dracena draco*<sup>24</sup>. Shoji's group isolated ophiogonanone A (30)<sup>25</sup> and methylophiopogonanones A (31) and B (32) (plus related chromones) from *Ophiopogon japonicus*<sup>26</sup>. Later chromanones (33)–(37) were isolated from the same source<sup>27</sup>. Compounds (31)–(34) were also isolated from *O. ohwii*, along with two related chromones. The same paper reported the isolation of (38) from *O. jaburan* plus three novel chromones<sup>28</sup>. Five new metabolites (39)–(43) were isolated from *Muscari comosum*, along with the already known (15)<sup>29,30</sup>.

Dewick has shown that eucomin incorporates radioactivity from tyrosine, and that the "extra" carbon atom, the ring 2-carbon, is methionine-derived<sup>31</sup>. The [6'-O-methyl-<sup>14</sup>C]-chalcones (44) and (45) were synthesised, and both were incorporated into eucomin, with the latter having the higher incorporation<sup>32</sup>. Thus the term "homoisoflavanoid" is misleading from a biogenetic viewpoint, and the alternative term "benzylchromanone" has been proposed. The proposed mechanism is similar to that of the heterocyclic ring formation in rotenoid biosynthesis (scheme 1).

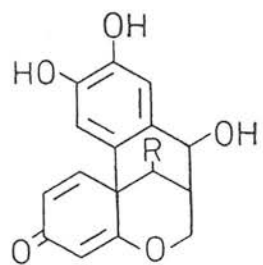
In 1973 the spiro compounds scillascillin (47) and its 2-hydroxy-7-O-methyl analogue (46) were isolated from *Scilla scilloides* along with 3,9-dihydroautumnalin (15)<sup>33</sup>. Dewick has suggested a derivation of (46) from a suitably activated 3-hydroxy-3-benzyl-4-chromanone such as (48) (scheme 2)<sup>31</sup>. A similar spiro-benzcyclobutene compound (49) was isolated from *M. comosum*, along with a possible isoflavanone-type derivative, comosin (50), and (51), the first example of an O-acetyl homoisoflavanoid, and already known (17)<sup>34</sup>. However, the substitution pattern at the 3' and 4' positions of (49) and (50) make their structures inconsistent with the above mechanism; it is possible that these structures have been wrongly assigned. Also the mechanism does not readily allow for the formation of (48).



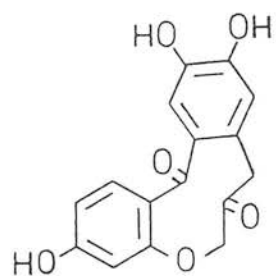
(51)



(52)



(53) R=OMe

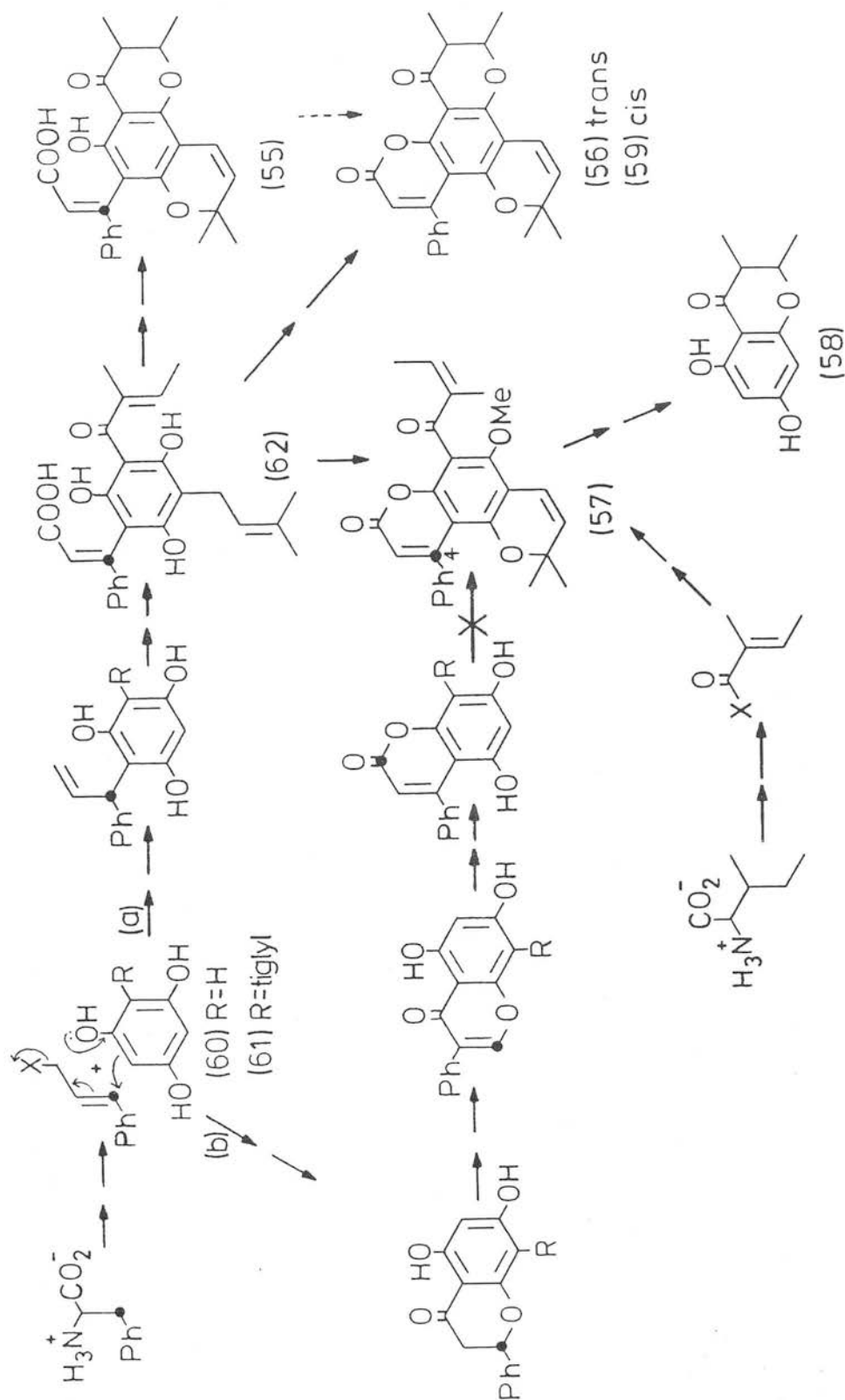


(54)

Instead of immediate formation of a fused benzcyclobutane intermediate, with participation of the meta- hydroxy group, a possible alternative route could involve the formation of a spirocyclopropylbenzenonium ion, with participation of the para- alkoxy group (the existence of this type of species is discussed in Section 2.1.2). Such a cation could further elaborate to (47); quenching with acetate would lead to formation of (50).

Brasilin (52), a metabolite of brasilwood<sup>35</sup>, may be derived from a homoisoflavanoid. It was later isolated from *C. sappan*, along with (53) and (54), which also appear related biosynthetically<sup>36</sup>. Homoisoflavanoids were reviewed by Tamm in 1981<sup>37</sup>.

After completion of this manuscript, 2 new homoisoflavanones isolated from *C. sappan* were reported<sup>107</sup>.

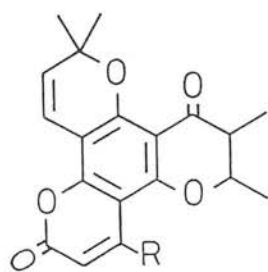




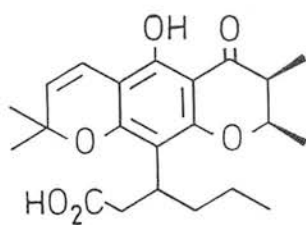
### 1.2.2. 2,3-Dimethyl-4-chromanones

Tropical trees of the *Calophyllum* (*Guttiferae*) genera are known to produce 2,3-dimethylchromanones. Nearly all are oxygenated at C-5 and C-7; the remaining (6- and 8-) positions are occupied by a prenyl substituent (which may be cyclised to a chromene), and an acid linked through its  $\beta$ -carbon. These acids are formally derived from cinnamic acid, or from n-alkanoic acid homologues, and in some cases are cyclised to the corresponding lactones. In 1957 calophyllolic acid (55) and inophyllolide (56) were isolated from the bark of *C. inophyllum*, along with a related metabolite, calophyllolide (57), which upon degradation afforded the chromanone (58)<sup>38</sup>. Compounds such as inophyllolide are more often classified as coumarins or chromenes than chromanones. In 1968 two chromanones were isolated from the leaves of *C. inophyllum*. One was shown to be (+)-inophyllolide (rather than the racemate isolated previously), with the 2- and 3- methyl groups in a *trans*-disposition (56). The other chromanone was the *cis*- isomer (59)<sup>39</sup>. Three related chromanols were also isolated<sup>40</sup>.

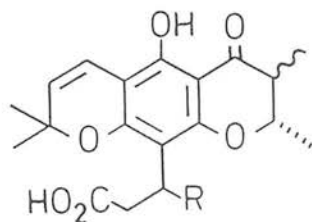
In 1967 Polonsky incorporated [3-<sup>14</sup>C]-phenylalanine into calophyllolide; degradation studies showed that most of the label was incorporated specifically into the 4- position (scheme 3). This important result precluded the biosynthesis of (57) from flavanones via one or more aryl-shifts, as shown in route (b). The result supports the pathway outlined in route (a), in which a phloroglucinol (60/61) is condensed with a cinnamic acid derivative<sup>41</sup>. [U-<sup>14</sup>C]-isoleucine was incorporated into the five carbon atoms of the chromanone portion of (57), presumably via tigloyl CoA or a related compound<sup>42</sup>. It is possible that tigloyl CoA acts as a "starter unit" for three malonate molecules in the biosynthesis of "tiglylphloroglucinol"(61), which then condenses with cinnamic acid and isoprene derivatives to form an intermediate



(63) R=Ph  
(64) R=C<sub>3</sub>H<sub>7</sub>

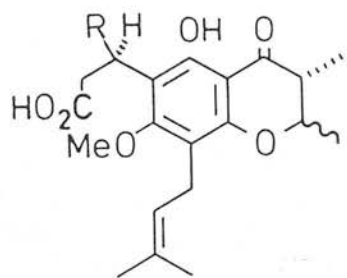


(65)

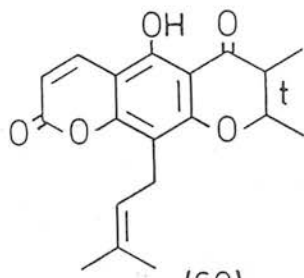


trans cis

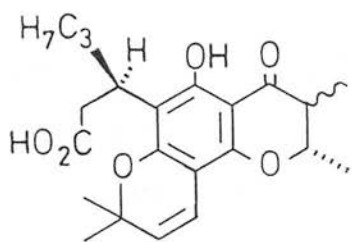
Ph	(71)	(82)
C <sub>5</sub> H <sub>11</sub>	(66)	(76)
C <sub>4</sub> H <sub>9</sub>	(77)	(78)
C <sub>3</sub> H <sub>7</sub>	(70)	(79)
CH <sub>3</sub>	(80)	



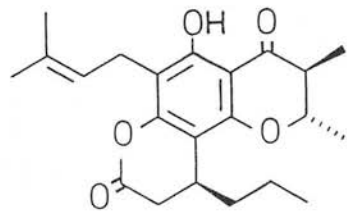
R trans cis  
C<sub>5</sub>H<sub>11</sub> (67) (68)  
C<sub>3</sub>H<sub>7</sub> (74)



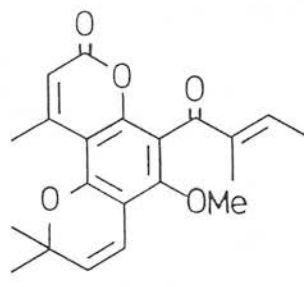
(69)



(72) trans  
(73) cis



(75)

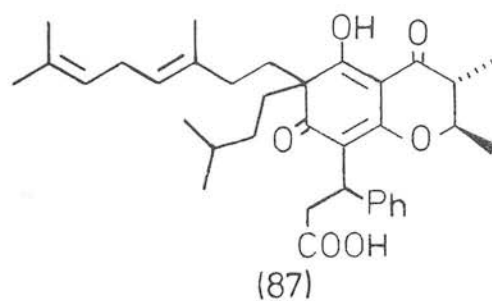
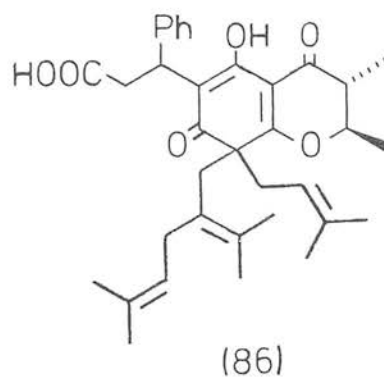
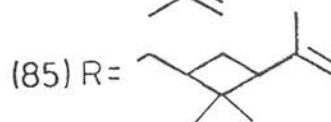
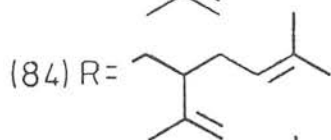
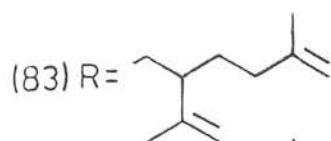
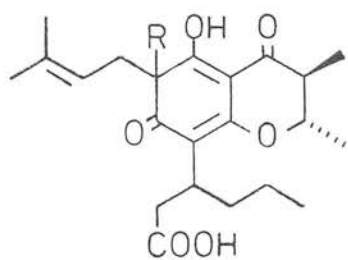


(81)

such as (62). In 1972 Polonsky reported the intact incorporation of [3- $^{14}\text{C}$ ]-phenylalanine into inophyllolide (55) and calophyllolic acid (56), with incorporation into the former eight times that of the latter, thus suggesting that (55) is a precursor to (56)<sup>43</sup>.

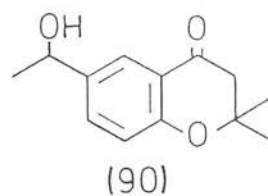
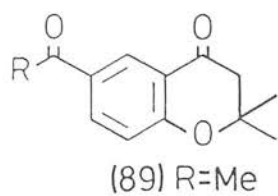
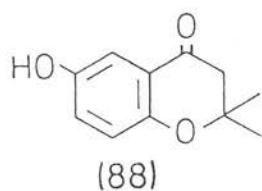
*C. tomentosum* produced two related metabolites, tomentolides A (63) and B (64). The former is an regioisomer of inophyllolide, and the latter contains a  $\beta$ -hexanoic acid substituent lactonised onto the C-7 hydroxy position. Apetalolide, a non-chromanone derivative of (64), was isolated from *C. apetalum*<sup>44</sup>. This also produced apetalic acid, reported as the *cis* chromanone (65)<sup>45</sup>. A *trans* homologue, blancoic acid (66), was isolated from *C. blancoi* by Stout and coworkers in 1968<sup>46</sup>. The related metabolites papuanic acid (67) and its 2-epimer, isopapuanic acid (68) were isolated from *C. papuanum*<sup>47</sup>. Compound (69) was isolated from *C. australianum*<sup>48</sup>; it was later isolated from *C. inophyllum* and named calaustralin<sup>49</sup>. In 1971 the previously known apetalic acid was isolated from *C. chapelieri*, along with its C-2 epimer, isoapetalic acid (70), dihydroisopetalic acid and chapelieric acid (71), which contains a phenylpropanoic acid moiety at the 8-position<sup>50</sup>.

Calolongic acid (72) was isolated from *C. calolongum*<sup>51</sup>; it was later isolated from *C. recedens*, along with its 3-epimer, isocalolongic acid (73), recedensic acid (74), a lower homologue of (64), and recedensolide (75)<sup>52</sup>. Six compounds (66), (70) and (76)–(79) were isolated from *C. brasiliense* in 1974<sup>53</sup>. The compounds were shown to be three epimeric pairs of homologous compounds containing octanoic, heptanoic and hexanoic acid moieties at the 8-position. A lower *trans* homologue, cordato-oblongic acid (80), was isolated from *C. cordato-oblongum*<sup>54</sup>. The same species yielded a related compound (81) and two related epimeric chromanols<sup>55</sup>. Isoapetalic acid (70) was subsequently isolated from *C. cuneifolium*<sup>56</sup>, and from *C. bracteatum*,



*C. calaba* , *C. moonii* and *C. trapezifolium* , along with its regioisomer, and the enantiomer of apetalic acid (probably 79) <sup>57</sup>. Chapelieric acid (71), was later isolated from *C. calaba* , along with its 2-epimer, isochapelieric acid (82)<sup>58</sup>.

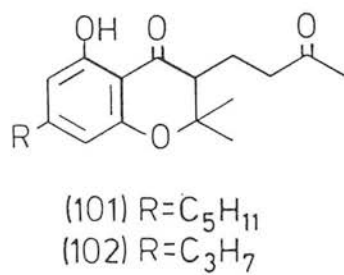
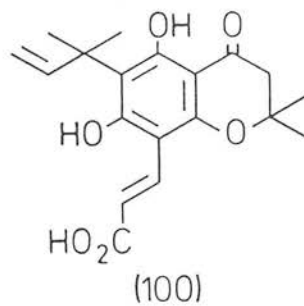
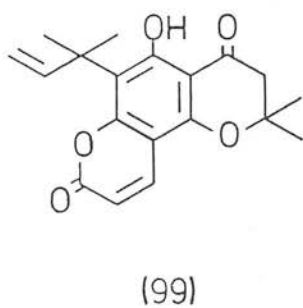
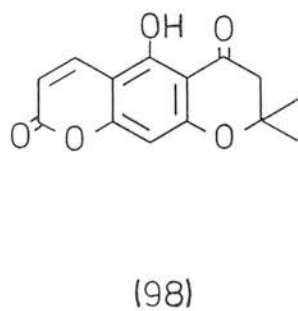
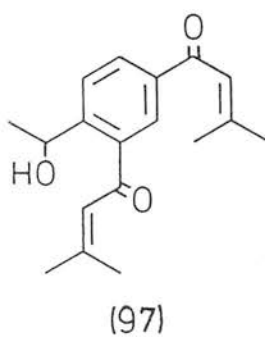
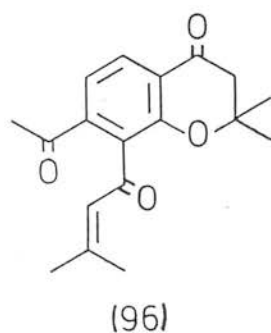
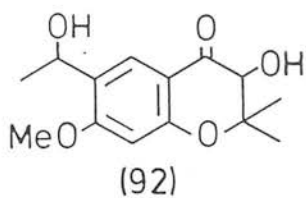
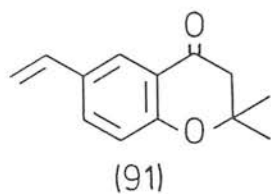
A group of nonaromatic, cyclohexadienone, compounds possibly related to some of the above have been reported. They contain a 7-oxo moiety, and the prenyl-bearing aromatic carbon has now been quarternised by the addition of a variety of ten-carbon substituents. Brasiliensic acid (83) and isobrasiliensic acid (84), isolated from *C. brasiliense* , and inophylloidic acid (85), isolated from *C. inophyllum* , were first reported in 1968<sup>59</sup>, and their structures later reassigned to those shown<sup>60</sup>. A further example, calophynic acid (86) was isolated from *C. inophyllum* <sup>61</sup>, and calozeylanic acid (87) was isolated from *C. zeylanicum* , *C. thwaitesii* and *C. walkeri* <sup>62</sup>.



(93) R=OH

(94) R=CH<sub>2</sub>OH

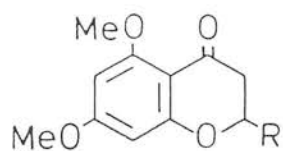
(95) R=CH<sub>2</sub>OAc



(102) R=C<sub>3</sub>H<sub>7</sub>

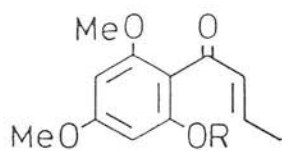
### 1.2.3. 2,2-Dimethyl-4-chromanones

Of the remaining naturally-occurring chromanones, most are 2,2- dimethyl compounds. The simplest of these, isolated from *Calea cuneifoli* , is 2,2-dimethyl-6-hydroxy-4-chromanone (88)<sup>63</sup>. The 6-acetyl compound (89) was isolated in 1977 from *Ambrosia cumanensis* <sup>64</sup>. Its dihydro-analogue (90), and the corresponding alkene (91), were isolated from *Trichogonia grazielae* , along with related non-chromanones <sup>65</sup>. Compounds (89) and (90) were later isolated from *Artemisia campestris* <sup>66</sup>, and (90) from *Doronicum grandiflorum* <sup>67</sup>. Its 3-hydroxy-7-methoxy derivative (92) was isolated from *Lagascea rigida* <sup>68</sup>. Three chromanones (93)–(95), also containing C-6 substituents, were isolated from *Chrysothamnus viscidiflorus* <sup>69</sup>. Dehydroisostoebenone (96) was isolated from *Stoebe plumosa* <sup>70</sup>. It was synthesised *in vitro* from its monocyclic diketone co-metabolite (97). Clausenin (98) and clausenidin (99) were isolated from *Clausena heptaphylla* <sup>71</sup>. The related compound (100) was isolated from *C. excavata* <sup>72</sup>. Cannibachromanone (101) was identified as a component of a cannabis-containing cigarette<sup>73</sup>. The lower homologue (102) was also identified later<sup>74</sup>.



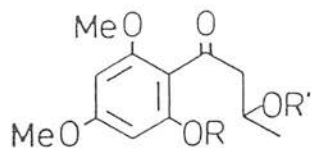
(103) R=Me

(104) R=Et



(105) R=Me

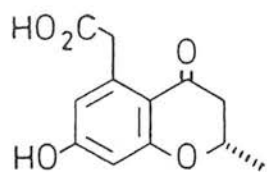
(109) R=H



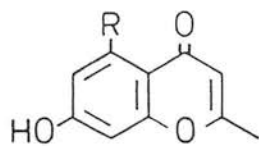
(106) R=H, R'=β-D-allosyl

(107) R=R'=H

(108) R=Me, R'=H



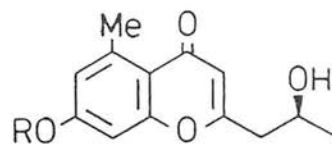
(110)



(111) R=CH<sub>2</sub>CO<sub>2</sub>H

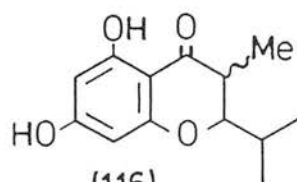
(112) R=Me

(113) R=CH<sub>2</sub>COMe

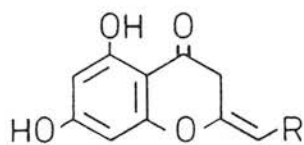


(114) R=H

(115) R=glucosyl

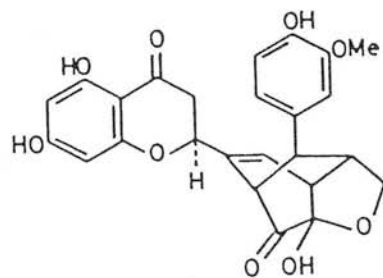


(116)



(117) R= (CH<sub>2</sub>)<sub>13</sub>CH<sub>3</sub>

(118) R=CH<sub>2</sub>CH<sub>2</sub>(CH=CHCH<sub>2</sub>)<sub>5</sub>CH<sub>3</sub>



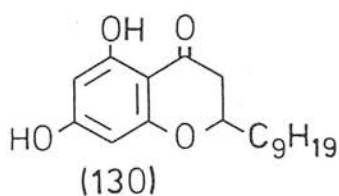
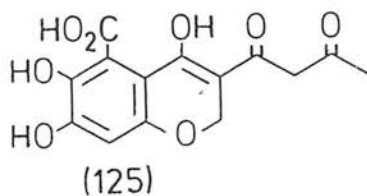
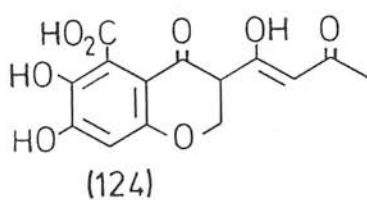
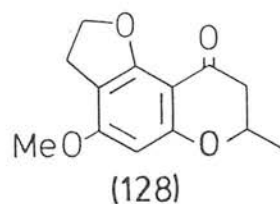
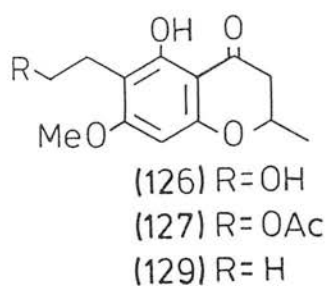
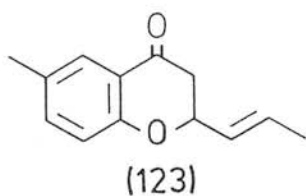
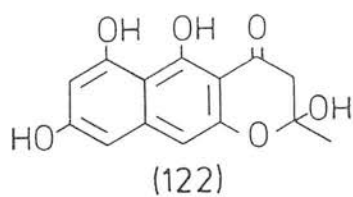
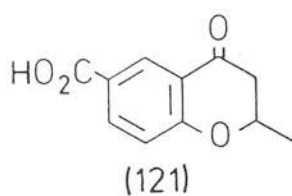
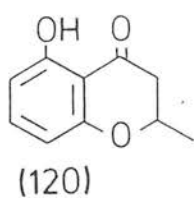
(119)



#### 1.2.4. Miscellaneous plant chromanones

The 2-methylchromanone eugenin (103) and its ethyl homologue (104) were isolated from the fern *Arachanoides standishii*, along with a related chalcone (105), and saturated metabolites (106),(107) and (108)<sup>75</sup>. Eugenin was later isolated from the herb *Dysophilla tometosa*, along with the corresponding chromone and the uncyclised chalcone (109), and its saturated analogue, an arylbutyrophenone<sup>76</sup>. The chromanone (110) was isolated from *Rheum* (rhubarb) rhizomes, along with five chromones (111)-(115). The authors suggested a biogenesis from degraded hexa- and heptaketides<sup>77</sup>.

Both 3-epimers of the 2-isopropylchromanone (116) were isolated from *Helichrysum bellum*<sup>78</sup>. Singly and polyunsaturated side-chains are present respectively in (117) and (118), isolated from the seaweed *Zonaria tournefontii*<sup>79</sup>. Silymonin (119), isolated from *Silybum marianum*, contains an unusual bicyclic substituent at the chromanone 2-position<sup>80</sup>. Although it is *formally* derived from a Diels-Alder addition between a 5,7-dihydroxyflavanone and coniferol, no biosynthetic information is available.



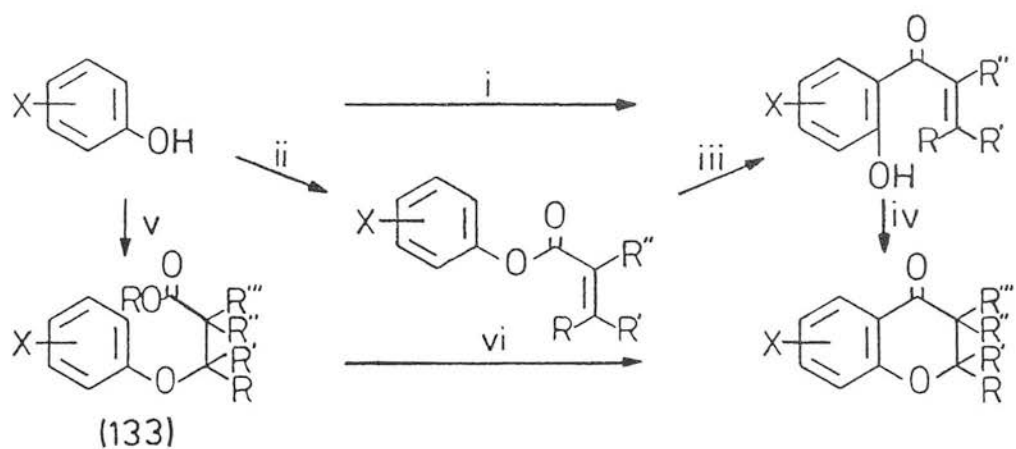
### 1.2.5. Fungal Chromanones

Of the fungal derived chromanones<sup>81</sup>, the first isolated was 5-hydroxy-2-methyl-4-chromanone (120), a metabolite of *Daldinia concentrica*, an ash parasite. A polyketide origin was deduced from radiolabelling studies<sup>82</sup>. Rosellinic acid (8-hydroxy-2-methyl-4-chromanone-6-carboxylic acid) (121) was isolated from *Rosenillia pecatrix* in 1964<sup>83</sup>. Fonsecin (122), a naphthopyranone, and its methyl ether, were isolated from an *Aspergillus fonscaeus* mutant<sup>84</sup>. It has also been identified as a component of certain dimers derived from *A. awamori*, *A. fonscaeus* and *A. niger*<sup>85</sup>. Structurally it is closely related to rubrofusarin (7), an established heptaketide chromone. In 1972 myrochromanone (123), and the corresponding chromanol, were isolated from *Myrothecium roridum*<sup>86</sup>. These, as does rosellinic acid, contain a carbon substituent at the 6- position and is lacking in the 5,7- $\beta$ - oxidation pattern associated with polyketide derived phenolic fungal metabolites. Polivione, a metabolite of *Penicillium frequentans*, has been shown to exist as a mixture of tautomers (124) and (125)<sup>87</sup>. In 1972 three chromanone metabolites were isolated from *Phoma pigmentivora* by McGahren and coworkers<sup>88</sup>. The major metabolite, designated LL-D253 $\alpha$ , was assigned structure (126), 5-hydroxy-6-(2-hydroxyethyl)-7-methoxy-2-methyl-4-chromanone. Two minor metabolites, LL-D253 $\beta$  and LL-D253 $\gamma$ , were assigned structures (127) and (128) respectively. LL-D253 $\alpha$  was subsequently isolated from *Phoma* strain NHL 7017<sup>89</sup>, and from *Sclerotinia fructigena*. The same three metabolites were obtained from *P. violacea*, along with the related ethyl chromanone (129)<sup>90</sup>. The structure of (126) was subsequently reassigned (see following chapter).

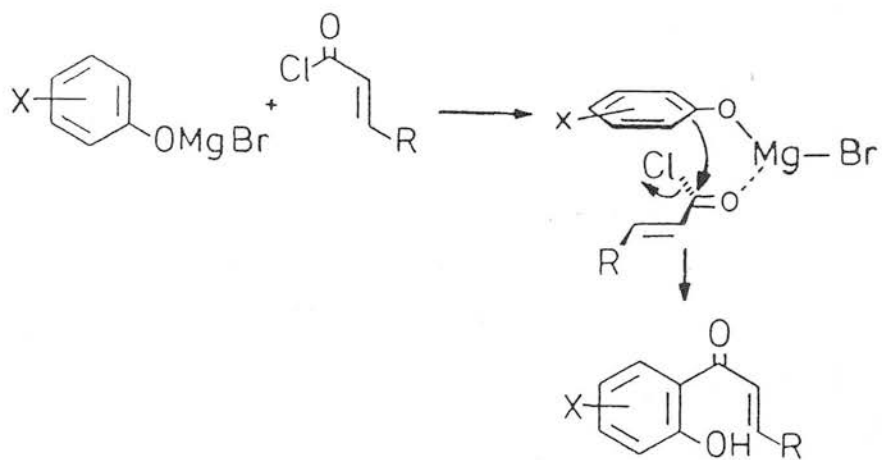
### 1.2.6. Other Naturally-Occurring Chromanones

Recently 5-hydroxy-2-nonyl-4-chromanone (130) and related metabolites were isolated from the secretory hairs of the azalea lace bug (*Stephantia*

*pyrivides* ) nymph<sup>91</sup>.



Scheme 4: i) Friedel-Crafts acylation; ii) Esterification;  
 iii) Fries Rearrangement; iv) Base; v) Ether Formation;  
 vi) Polyphosphoric acid.



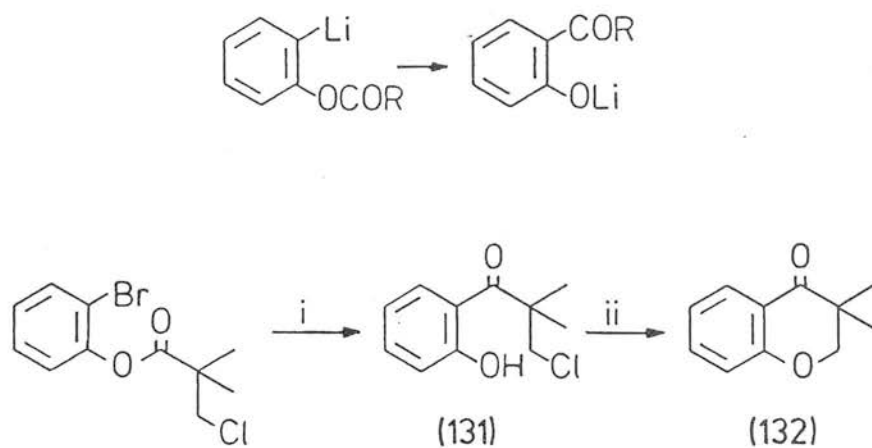
Scheme 5

### 1.3. Synthetic Routes to 4-Chromanones

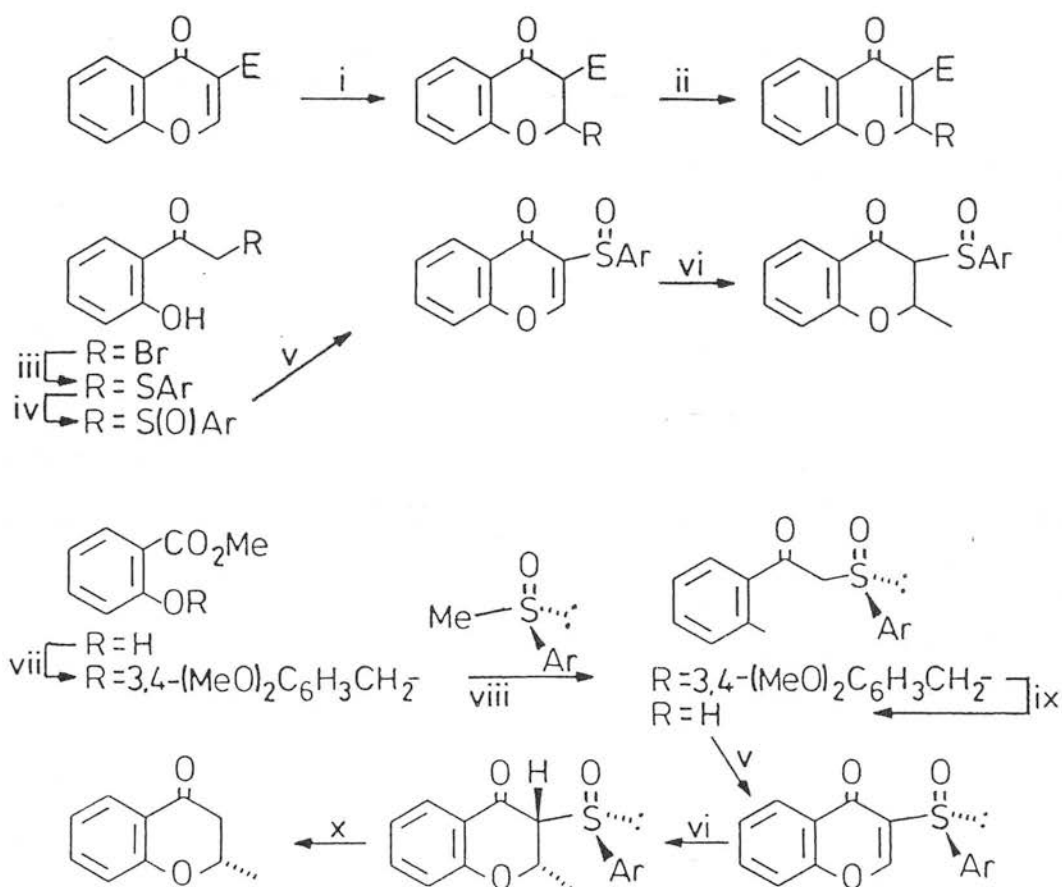
The most important methods for synthesising 4-chromanones are well-established and have been comprehensively reviewed<sup>2,3,92,93</sup>. For these reasons only a brief account will be given here, concentrating on more recent developments.

The most widely used method involves the Friedel-Crafts reaction, or the related Fries rearrangement<sup>94</sup>. These are particularly useful for the synthesis of 2-substituted chromanones. In the Friedel-Crafts reaction, a phenol, or in a few cases its methyl ether, is reacted with an unsaturated acid chloride or anhydride (or less frequently with the parent unsaturated acid) in the presence of a Lewis acid catalyst. The resulting unsaturated phenol may cyclise during the course of the reaction, or work-up, but may also be isolated and subsequently cyclised in the presence of alkali (scheme 4).

A variety of catalysts have been used successfully; these include aluminium trichloride, boron trifluoride, hydrofluoric acid and polyphosphoric acid (which also effects cyclisation to the desired chromanone). The most commonly used solvent has been nitrobenzene. A "thermal condensation" in the absence of catalyst or solvent has been reported in the synthesis of 5,7-dihydroxy-2,2-dimethyl-4-chromanone<sup>95</sup>. More recently phosphorus pentoxide and methanesulphonic acid have been used in a one-step synthesis of 2,2-dialkyl 4-chromanones<sup>96</sup>. Another interesting development has been the "magnesium directed Friedel-Crafts reaction", in which a magnesium phenolate is reacted with acyl chlorides to give almost exclusively ortho acylation<sup>97</sup>. This regioselectivity has been attributed to the association between the aryloxy-bound magnesium and the carbonyl carbon of the electrophile (scheme 5).



Scheme 6. Reagents: i) *sec* BuLi,  $H^+$ ; ii) NaH.



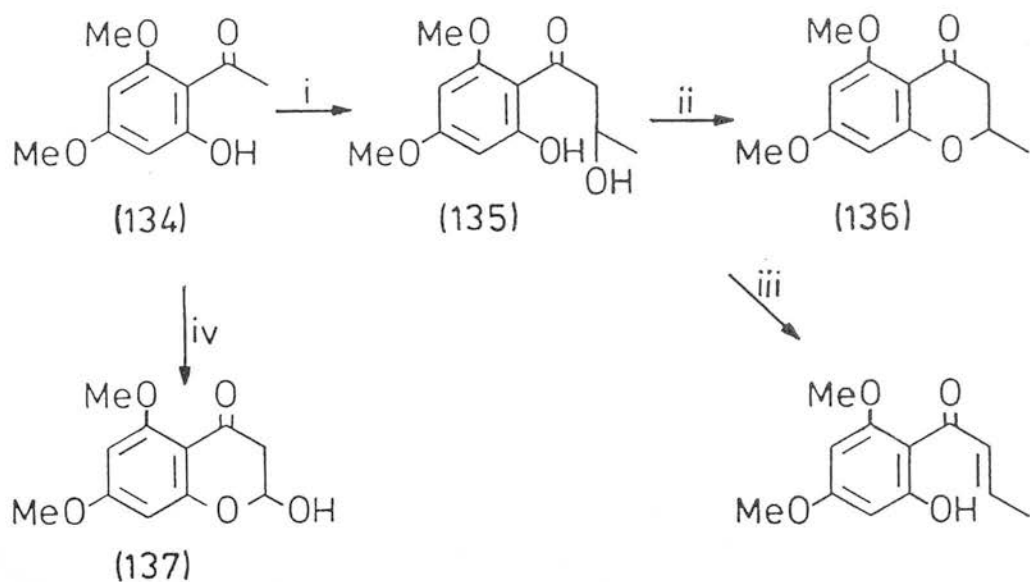
Scheme 7. E = Electron-withdrawing group; Ar = *p*-MeC<sub>6</sub>H<sub>4</sub><sup>-</sup>.  
 Reagents: i) RM; ii) DDO; iii) ArSH, EtOH; iv) *m*-CPBA; v) AcOCHO, HCO<sub>2</sub>Na;  
 vi) Me<sub>2</sub>CuLi; vii) 3,4-(MeO)<sub>2</sub>C<sub>6</sub>H<sub>3</sub>CH<sub>2</sub>Cl, K<sub>2</sub>CO<sub>3</sub>;  
 viii) LDA; ix) Ph<sub>3</sub>C<sup>+</sup>BF<sub>4</sub><sup>-</sup>; x) a) Al(Hg), b) PDC.

The related Fries rearrangement has been used to construct 4-chromanones by heating the appropriate unsaturated phenoxy ester in the presence of a Lewis acid (scheme 4). Recent developments have seen the use of the *photo*-Fries rearrangement. Further refinements include a two-phase system<sup>98</sup> and the addition of potassium carbonate<sup>99</sup>, to increase product yields. Recently, a "metal-promoted" Fries rearrangement was reported in the synthesis of (131), which was cyclised to 3,3-dimethyl-4-chromanone (132) (scheme 6)<sup>100</sup>.

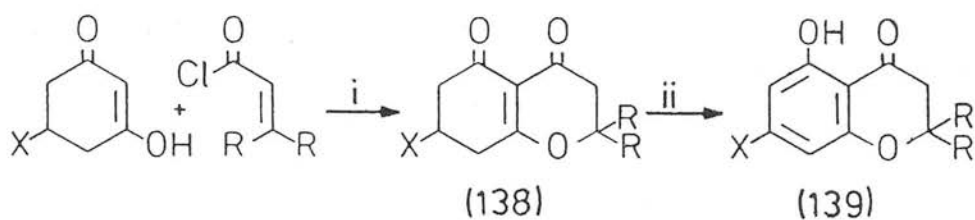
The next most successful method has involved the cyclisation of 3-phenoxy propionic acids (133), in the presence of a catalyst or dehydrating agent such as phosphorus pentoxide, polyphosphoric acid, hydrogen fluoride or sulphuric acid. The related 3-phenoxypropionoate esters or 3-hydroxypropionitriles have also been used (scheme 4)<sup>101</sup>.

The remainder of the established methods have been of lesser importance than those mentioned above. The double-bond of a chromone may undergo addition to give a chromanone. This is useful if the former is synthetically more accessible than the latter. The most common example is catalytic hydrogenation. This however cannot lead directly to 2,2- or 3,3- disubstituted chromanones. Other species such as bromine may be added across the double bond. Recently conjugate addition of organocuprate species to chromones has been reported; this results in 2-substitution and should prove synthetically useful as chromones are generally more accessible than chromanones. Good yields are obtained only when the 3-position is activated, but 3-unsubstituted chromanones may be obtained via temporary activation with, and subsequent removal of, a carbomethoxyl moiety<sup>102</sup>. The use of a chiral activating group, such as tolylsulphonyl, has made possible chirally-induced conjugate addition, leading to asymmetric syntheses with respect to the 2-position (scheme 7)<sup>103</sup>.





Scheme 8. Reagents: i) a) LDA, b)  $\text{CH}_3\text{CHO}$ ; ii)  $\text{H}^+$ ; iii) pTSA; iv) NaH,  $\text{HCO}_2\text{Me}$ .



Scheme 9. Reagents: i)  $\text{TiCl}_4$ ; ii) a) Pd/C or b) DDQ or c) NBS,  $(\text{PhCO}_2\text{O})_2$ .

The condensation of o-hydroxyacetophenones with aldehydes has been mainly limited to benzaldehydes, leading to flavanones. Recently however the lithium enolate of 2'-hydroxy-4',6'-dimethoxyacetophenone (134) was condensed with acetaldehyde to give (135) which was cyclised to 5,7-dimethoxy-2-methyl-4-chromanone (136)<sup>104</sup>. The same acetophenone was condensed with methyl formate in the presence of sodium hydride to give the corresponding 2-hydroxy-4-chromanone (137) (scheme 8)<sup>105</sup>. Chromanones have been obtained from oxidation of chromans and chromanols, but these are of little synthetic use as they are usually prepared from chromanones.

A novel approach to 5-hydroxy-4-chromanones involves the condensation of 1,3-cyclohexanediones with unsaturated acid chlorides in the presence of titanium tetrachloride. The product (138) may readily be aromatised to (139) by standard methods (scheme 9). This procedure is regioselective, whereas the conventional use of resorcinol derivatives would yield a mixture of 5- and 7-hydroxy-4-chromanones<sup>106</sup>.

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1. F.M. Dean, "Naturally-occurring Oxygen Ring Compounds", Butterworths, London, 1963, p248-266.
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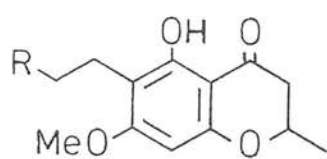
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## CHAPTER 2

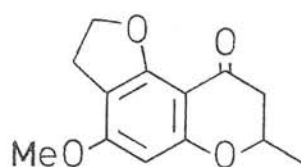
**SYNTHESIS AND BIOSYNTHESIS OF LL-D253 $\alpha$   
AND ITS DERIVATIVES**



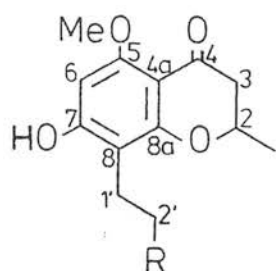
(1) R=OH

(2) R=OAc

(4) R=H



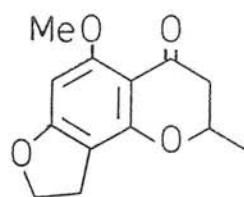
(3)



(5) R=OH

(6) R=OAc

(8) R=H



(7)

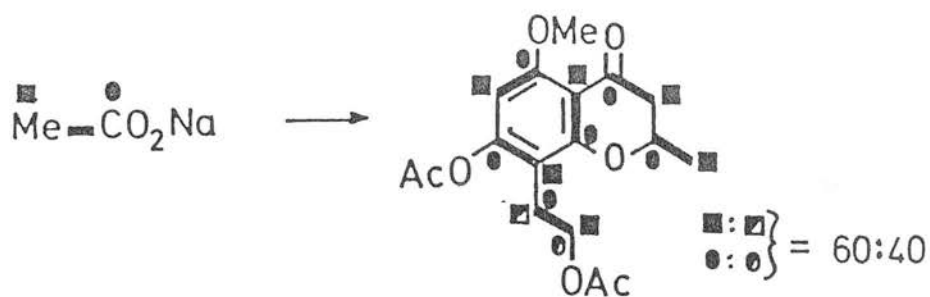
## 2.1. Introduction

### 2.1.1. Previous Work

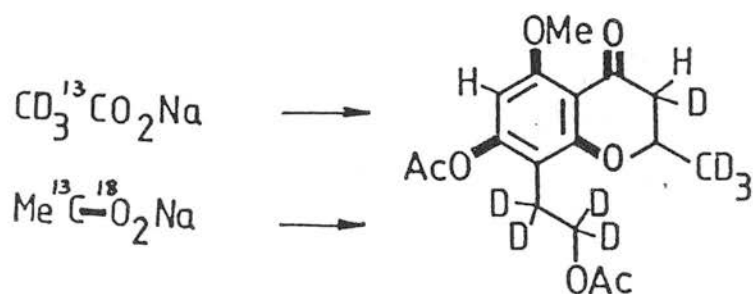
Inspection of the initially-reported structures<sup>1,2,3</sup> of the four *Phoma* metabolites (1)–(4) would suggest a polyketide origin for the chromanone nucleus, but the source of the two-carbon side-chain is not readily apparent. For this reason McIntyre and Simpson isolated LL-D253 $\alpha$  (1), in order to study its biosynthetic origin<sup>4</sup>. However it soon became clear, mainly from  $^{13}\text{C}$  n.m.r. studies, that the published structure of (1) was incorrect. An alternative structure was proposed, principally on the basis of high field  $^{13}\text{C}$  n.m.r. decoupling experiments. This structure (5) was confirmed by the synthesis of both the new, and the originally proposed, structures<sup>5</sup>. For the purposes of this discussion it has been assumed that the minor metabolites (2), (3) and (4) are reassigned as (6), (7) and (8) respectively, but it must be stressed that this remains to be proven.

A series of  $^{14}\text{C}$ -labelled precursors were then administered to *Phoma pigmentivora*, in order to gain information on the source of the hydroxyethyl side-chain of LL-D253 $\alpha$ . Incorporations of labelled glycollate and mevalonate were negligible; labelled oxalate was incorporated efficiently, but degradation studies showed that the label was localised at the 5-methoxyl carbon. The only compound to be efficiently incorporated into the carbon skeleton of LL-D253 $\alpha$  was acetate.

*P. pigmentivora* was then fed with  $[1-^{13}\text{C}]$ -,  $[2-^{13}\text{C}]$ - and  $[1,2-^{13}\text{C}_2]$ -acetates<sup>6,7</sup>. Analysis of the resultant  $^{13}\text{C}$ - n.m.r. spectra of LL-D253 $\alpha$  showed the anticipated labelling pattern for the chromanone nucleus (scheme 1), confirming its polyketide origin. However the side-chain had appeared to



Scheme 1: Incorporation of  $[1-^{13}\text{C}]$ -,  $[2-^{13}\text{C}]$ - and  $[1,2-^{13}\text{C}_2]$ -acetates into LL-D253α

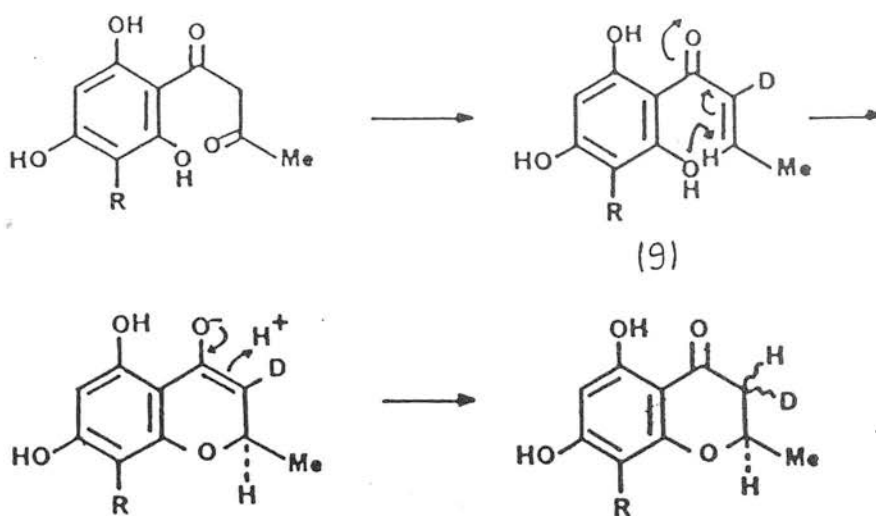


Scheme 2: Incorporation of  $[1-^{13}\text{C}, 2-^2\text{H}_3]$ - and  $[1-^{13}\text{C}, ^{18}\text{O}_2]$ -acetates into LL-D253α

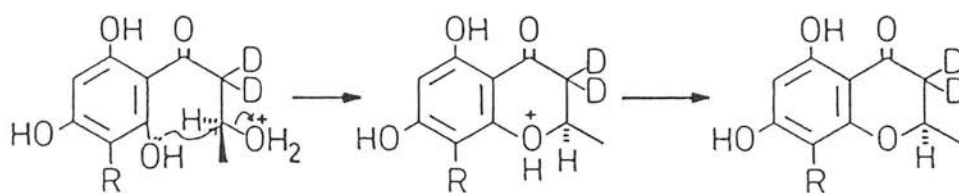


undergo a *partial* (80%) randomisation. The incorporation of  $[1-^{13}\text{C}, 2-^2\text{H}_3]$ -acetate into LL-D253 $\alpha$  gave isotopically-shifted  $^{13}\text{C}$  resonances<sup>8</sup> at C-2, C-4, and *both* C-1' and C-2', indicating the presence of deuterium at C-1, C-3, C-2' and C-1' respectively. The C-2 signal shows that up to three deuteriums are retained at C-1; thus these two carbons constitute the "starter unit" for the polyketide chain (scheme 2). No isotopically-shifted signal was observed at C-5. If deuterium were incorporated at C-6, it may have been lost due to exchange; there is precedent for preferential deuterium loss for analogous aromatic positions *ortho* to two hydroxyl groups<sup>9</sup>. Alternatively, a signal may be present, but is too small to be resolved<sup>10</sup>. The isotopically shifted signal at C-4 was not well resolved and it was not possible to determine whether one or two deuteriums were present in those molecules which carried a  $^{13}\text{C}$ -label at C-4. Incorporation of  $[2-^2\text{H}_3]$ -acetate into LL-D253 $\alpha$  gave a labelled species. Its 3-axial *and* 3-equatorial deuterium n.m.r. signals were resolved in the presence of a shift reagent, and these signals were of equal intensity. However it was not clear whether these signals were due to molecules containing both a 3-axial and a 3-equatorial deuterium label, or a mixture of singly (either axially or equatorially) deuteriated species.

The incorporation of  $[1-^{13}\text{C}, ^{18}\text{O}_2]$ -acetate<sup>11</sup> into LL-D253 $\alpha$  resulted in isotopically-shifted resonances at C-4, C-5, C-7 and C-8a, indicating the acetate-derivation of the attached oxygen atoms, and the integrity of these carbon-oxygen bonds during biosynthesis (scheme 2). Incorporation of  $^{18}\text{O}_2$  gas into LL-D253 $\alpha$  revealed that 20% of the oxygen at C-2' is derived from atmospheric oxygen, and none from acetate. The only other logical oxygen source is from the water of the growth medium. LL-D253 $\alpha$  is optically active, and has been assigned the 2R configuration<sup>1</sup>. Thus chromanone formation is stereospecific with respect to the 2-position.

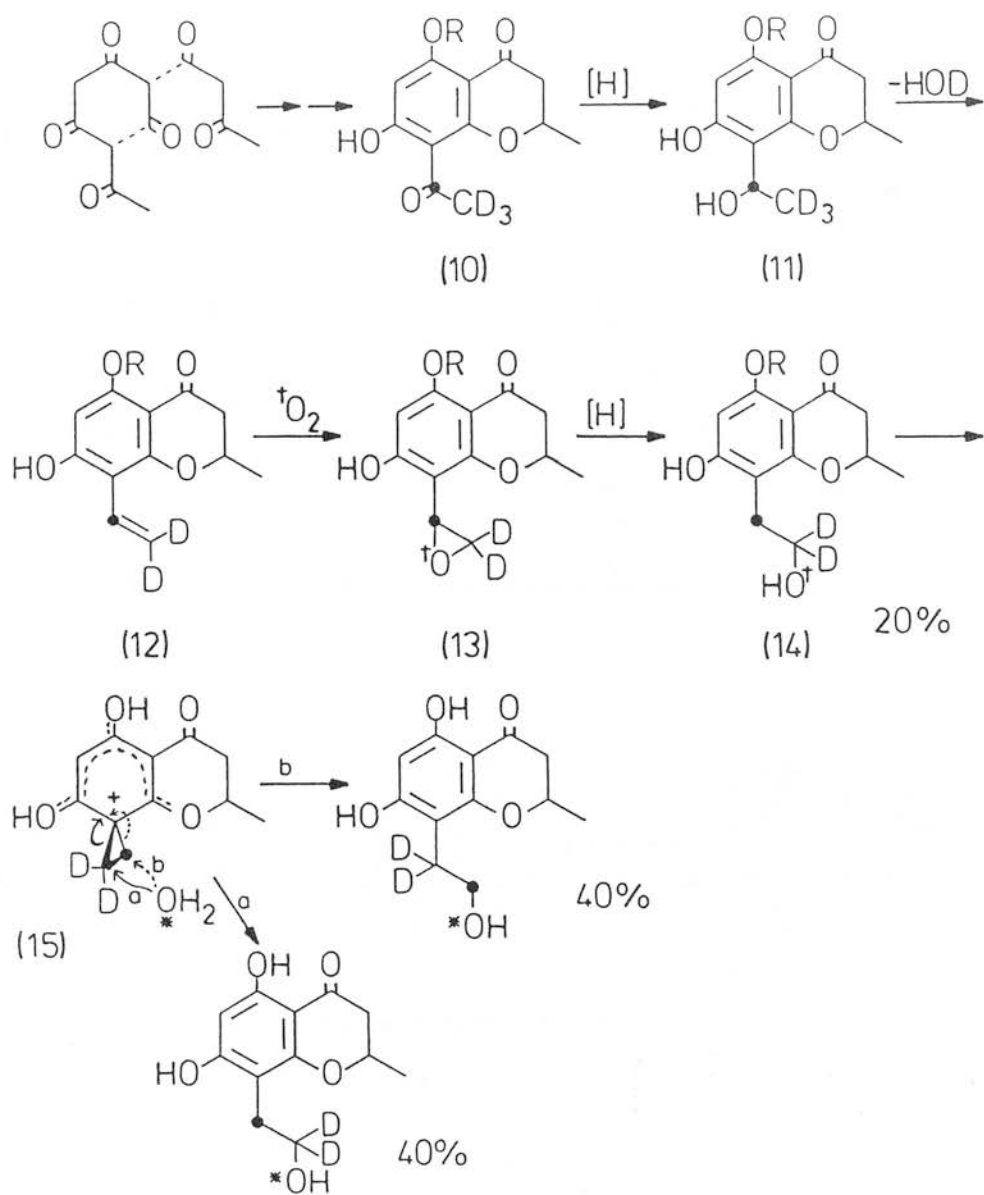


Scheme 3a: Only one deuterium retained at C-3



Scheme 3b: Two deuteriums retained at C-3

Scheme 3: Proposed mechanisms for pyranone ring formation in LL-D253 $\alpha$

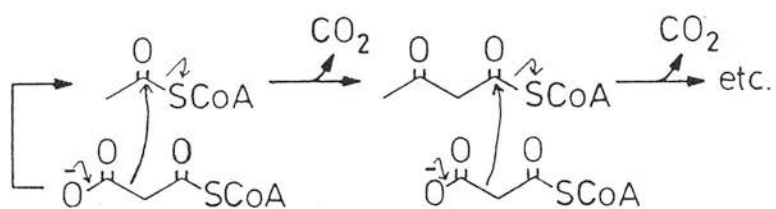
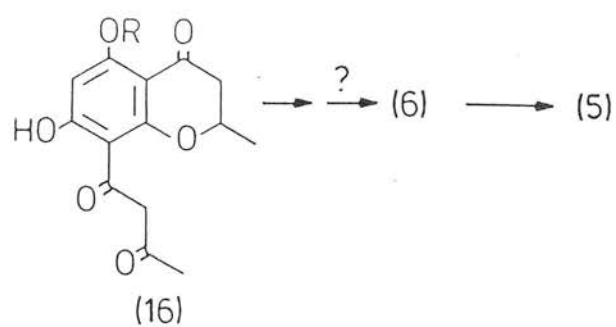


Scheme 4: Proposed biosynthesis of LL-D253 $\alpha$

From the above results, two mechanisms for formation of the pyranone ring may be envisaged; one involves conjugate addition of a phenolic oxygen to unsaturated ketone (9) (scheme 3a). The resulting enolate may then be protonated at the 3-position; if only one deuterium is retained at C-3 of LL-D253 $\alpha$ , then protonation of this enolate must occur equally from either face, to give a mixture of axially and equatorially deuteriated isotopomers. If however two deuteriums are retained at the C-3 position, then scheme 3a is not valid. An alternative mechanism (scheme 3b) may be envisaged, whereby the 4-carbon side-chain bears a hydroxyl moiety at C-2; this may be protonated, and displaced by the phenolic hydroxyl to form the chromanone ring. It was suggested that clarification of this could be obtained by the incorporation of [2- $^{13}\text{C}$ , $^2\text{H}_3$ ]-acetate into LL-D253 $\alpha$  and analysis of the  $\alpha$ -shifted<sup>12</sup> C-3  $^{13}\text{C}$ -n.m.r. signal could reveal how many deuterium atoms are present at the C-3 position. (See Discussion , Section 2.2.3).

To account for all of the above incorporation results it has been proposed<sup>4,13</sup> that two polyketide fragments (a C<sub>10</sub> plus a C<sub>2</sub>, a C<sub>8</sub> plus a C<sub>4</sub>, or two C<sub>6</sub> fragments) form an acetyl chromanone such as (10) (scheme 4), and this acetyl side-chain is then reduced, dehydrated, epoxidised and ring-opened to give the 2'-hydroxyethyl-chromanone (14). Of this, 80% forms a "spirocyclopropylbenzenonium" ion (15), by expulsion of hydroxide ion (or of water, if the intermediate is protonated), with participation of the aryl nucleus, as shown in scheme 4. This intermediate may then undergo nucleophilic ring opening at either the (acetate-) carboxyl-derived carbon (no rearrangement), or the (acetate-) methyl-derived carbon (net rearrangement). The existence of such an intermediate is considered below. The intermediacy of (12) may be used to rationalise the formation of the ethyl chromanone (8), by reduction.

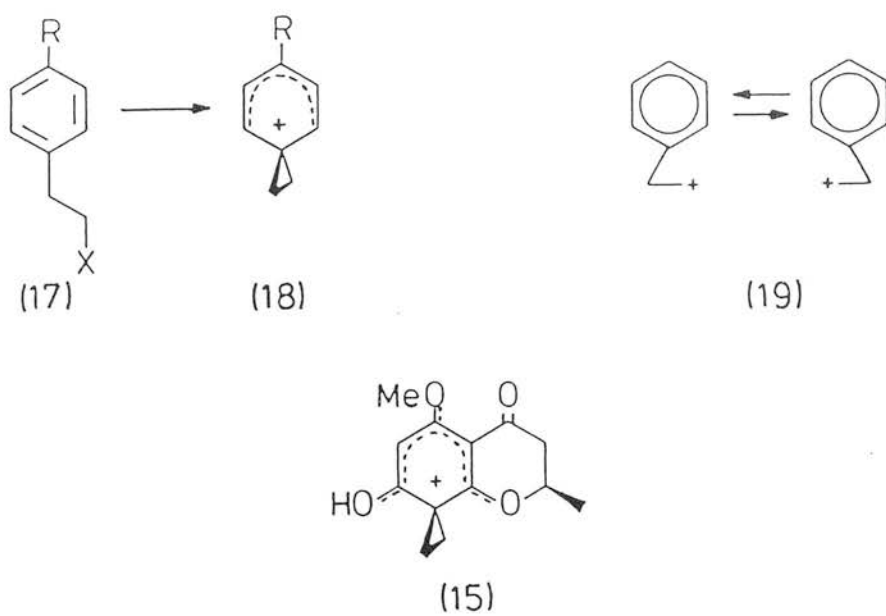
An intriguing possibility is that LL-D253 $\beta$  (6) is derived from an



Scheme 5: Generalised polyketide biosynthesis

*acetoacetate*-substituted intermediate (such as (16)), by a biological Baeyer-Villiger oxidation. If this were the case, then the acetate portion of LL-D253 $\beta$  should exhibit a similar degree of labelling, from labelled acetate precursors, as the carbon skeleton. Unfortunately this metabolite is produced by *P. pigmentivora* in very small quantities. A less direct approach would be to investigate the incorporation of labelled malonate into LL-D253 $\alpha$ .

In the biosynthesis of polyketides, acetyl CoA forms the "starter unit"<sup>14</sup> and the remainder of the polyketide chain is constructed by successive condensations of the existing chain with a molecule of malonyl CoA, and expulsion of carbon dioxide (scheme 5). Thus the chain elongates by two carbon atoms, but only the starter unit is derived directly from acetyl CoA. For this reason, incorporation of malonate into polyketides is, in some instances, observed to be lower at the starter unit than the remainder of the molecule, because malonate must first be converted to acetate, with some of the former being utilised in other biosynthetic processes. Thus if LL-D253 $\beta$  is derived from an *acetoacetate*-substituted chromanone, incorporation of malonate into the side chain should be similar to incorporation into the carbon skeleton. If however the side-chain starts as *acetate*, then the starter effect would be expected to depress incorporation of malonate at the C-1' and C-2' carbons.



Scheme 6

### 2.1.2. Phenonium ions

The solvolysis of  $\beta$ -arylethyl systems and the nature of their products has been important to the "non-classical ion" problem<sup>15,16</sup>, a topic of intense debate and experimental effort from the late 1940s. Broad agreement was reached only around 1970, but the topic still attracts attention<sup>17</sup>. Kinetic and stereochemical studies on the solvolysis of some  $\beta$ -arylethyl compounds (17)<sup>18</sup> supported the formation of a bridged symmetrical intermediate such as (18), termed a "spirocyclopropylbenzenonium" or "phenonium" ion. Subsequently, alternative structures were proposed involving a pair of rapidly equilibrating "carbenium" ions (19), or, such ions stabilised by through-space delocalisation by the aromatic  $\pi$ -electrons.

Eventually further experimental studies appeared to favour the existence of phenonium structures. This was supported in 1967 by the proton n.m.r. spectrum of the species produced by ionising (17; R=OMe, X=Cl) in aprotic superacid solution<sup>19</sup>. The signals strongly favoured a "phenonium" structure (18, R=OMe) (scheme 6).

The author noted that the para-methoxy substituent in particular, and (ortho- and para-) hydroxy or alkoxy substituents in general, promote aryl participation in phenonium ion formation. Thus the structure of LL-D253 $\alpha$  is consistent with the possibility of an *almost* symmetrical phenonium ion (15) being formed, causing partial randomisation of the side-chain.



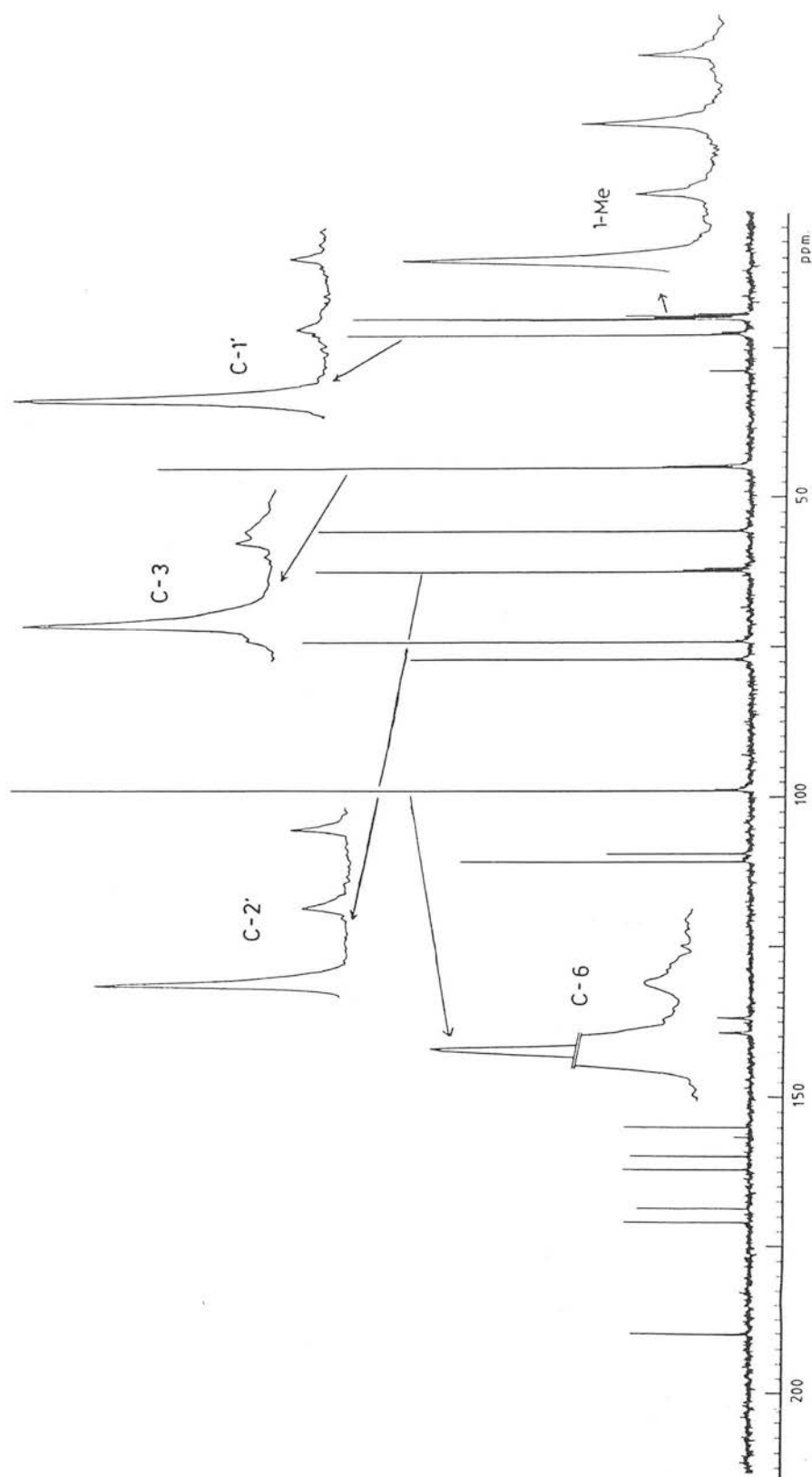


Figure 1: 100 MHz.  $^{13}\text{C}$  N.M.R. spectrum of LL-D253 $\alpha$  diacetate enriched from  $[2-^{13}\text{C}, 2^2\text{H}_3]$ -acetate.

Carbon	$\delta(\text{ppm})$	$\Delta\delta(\text{ppm} \times 100)$
2-CH <sub>3</sub>	20.4	26.9, 54.0, 81.1
-COCH <sub>3</sub>	20.6	
-COCH <sub>3</sub>	20.6	
1'	23.1	27.8, 55.7
3	45.4	32.6, 36.9
-OCH <sub>3</sub>	56.0	30.4, 61.0
2'	62.7	
2	74.1	29.8
6	98.9	
4a	109.4	
8	110.7	
7	155.1	
5	159.9	
8a	162.1	
-COCH <sub>3</sub>	168.6	
-COCH <sub>3</sub>	170.8	
4	190.1	

Table 1: <sup>13</sup>C n.m.r. spectrum of LL-D253 $\alpha$  diacetate derived from sodium [2-<sup>2</sup>H<sub>3</sub>,2-<sup>13</sup>C]-acetate.

## 2.2. Discussion

### 2.2.1. Summary

For the reasons described in the previous section, it was decided to study the incorporation of  $[2-^{13}\text{C}, ^2\text{H}_3]$ -acetate and  $[2-^{13}\text{C}]$ -malonate into LL-D253 $\alpha$ . In addition, it was proposed to investigate the *in vitro* feasibility of randomisation of its 2-carbon side-chain, as suggested in scheme 4. Finally it was hoped to synthesise each of the proposed intermediates (10)-(13), ultimately with a view to introduction of an isotopic label, in order to test their specific incorporation into LL-D253 $\alpha$ .

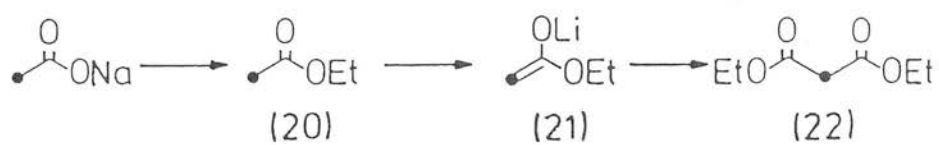
### 2.2.2. Formation of the Pyranone Ring of LL-D253 $\alpha$

It was decided that the two mechanisms for chromanone ring formation may be distinguished by incorporation of  $[2-^{13}\text{C}, ^2\text{H}_3]$ -acetate into LL-D253 $\alpha$ , and analysis of the resulting " $\alpha$ -shifted"  $^{12}$  nmr signals. Accordingly, this material was administered to *P. pigmentivora*. LL-D253 $\alpha$  was subsequently isolated, and its diacetate analysed by high field n.m.r. spectroscopy. Isotopically-shifted signals were observed at five resonances (Figure 1), those corresponding to C-1, C-3, C-6 and both C-1' and C-2' (see Table 1).

A total of three isotopically-shifted signals were observed at C-1, in agreement with the  $\beta$ -shift studies. Two deuteriums were present at each of C-1' and C-2', confirming that side-chain randomisation occurs<sup>14</sup>. The ratio of deuterium in originally labelled (5) to rearranged (5') material was 60:40, in agreement with previous results. Also worth noting is the novel observation of deuterium retention at C-6. Finally, two signals were observed for C-3, isotopically shifted by 0.326 and 0.369 ppm. Thus the magnitude of the shift

seems to show a slight stereochemical dependence. However there is no isotopically-shifted signal at around +0.70 ppm from the parent signal, which would be expected for molecules containing two deuteriums per labelled site. Thus the two signals observed are due to two different (axially and equatorially) monodeuteriated isotopomers. For this reason the ring-closure mechanism shown in scheme 3b may be ruled out. It is likely that the one shown in scheme 3a is valid, i.e. an enolate is formed stereospecifically, and then protonated, with equal facility, from either face. It is worth noting here that in the biosynthesis of flavanones from chalcones, the corresponding enolate is formed, but subsequent protonation *does* occur stereospecifically<sup>20</sup>.

The  $^2\text{H}$  spectrum of the above compound is also worthy of comment. Although peaks were broad, signals due to the 2-methyl, 3- and the 1'- and 2'-positions were apparent. All appeared to be split by coupling to  $^{13}\text{C}$ . This was clearest in the case of the sharper, more intense, methyl signal. Two peaks of equal intensity (1.24 and 1.60 ppm.) were observed, with a  $^2\text{H}$ - $^{13}\text{C}$  coupling constant of 19.5 Hertz. A less intense singlet at 1.45 ppm. is presumably due to the isotopomer containing deuterium connected to  $^{12}\text{C}$ . Interestingly, this signal is not exactly midway between the two peaks due to the  $^2\text{H}$ - $^{13}\text{C}$  doublet; thus it would appear that the  $^{13}\text{C}$  has induced an upfield isotope shift in the deuterium signal of 0.026 ppm. compared with deuterium connected to  $^{12}\text{C}$ . Although the other signals are less intense, it appears that for each of the three positions a doublet is present.



Scheme 7

Carbon	$\delta$ (ppm)	A	B	A/B	(A/B)*
2-CH <sub>3</sub>	20.40	7.306	5.530	1.321	1.4
-COCH <sub>3</sub>	20.60	6.189	4.865	1.272	1.7
-COCH <sub>3</sub>	20.67	6.537	4.865	1.343	1.6
1'	23.02	5.515	4.882	1.130	1.2
3	45.32	7.442	4.766	1.561	1.7
-OCH <sub>3</sub>	55.95	4.624	4.489	1.030	1.1
2'	62.57	5.875	4.813	1.221	1.3
2	73.96	4.922	5.306	0.928	1.0
6	98.77	7.141	4.245	1.682	1.8
4a	109.24	8.108	5.208	1.557	1.7
8	110.51	8.193	5.196	1.577	1.7
7	154.80	4.750	5.293	0.897	1.0
5	159.66	4.596	5.154	0.892	1.0
8a	161.89	4.822	5.572	0.865	0.9
-COCH <sub>3</sub>	168.43	4.795	4.575	1.048	1.1
-COCH <sub>3</sub>	170.68	4.421	5.088	0.869	0.9
4	189.92	4.765	4.658	1.022	1.1

Table 2: Comparison of signal intensities of the 91MHz. <sup>13</sup>C n.m.r. spectra of (A) LL-D253 $\alpha$  diacetate labelled from diethyl [2-<sup>13</sup>C]-malonate and (B) unlabelled LL-D253 $\alpha$  diacetate.

\* In the final column, the ratio of A to B is normalised to make the average ratio for the carbons derived from C-1 of acetate equal to 1.0.

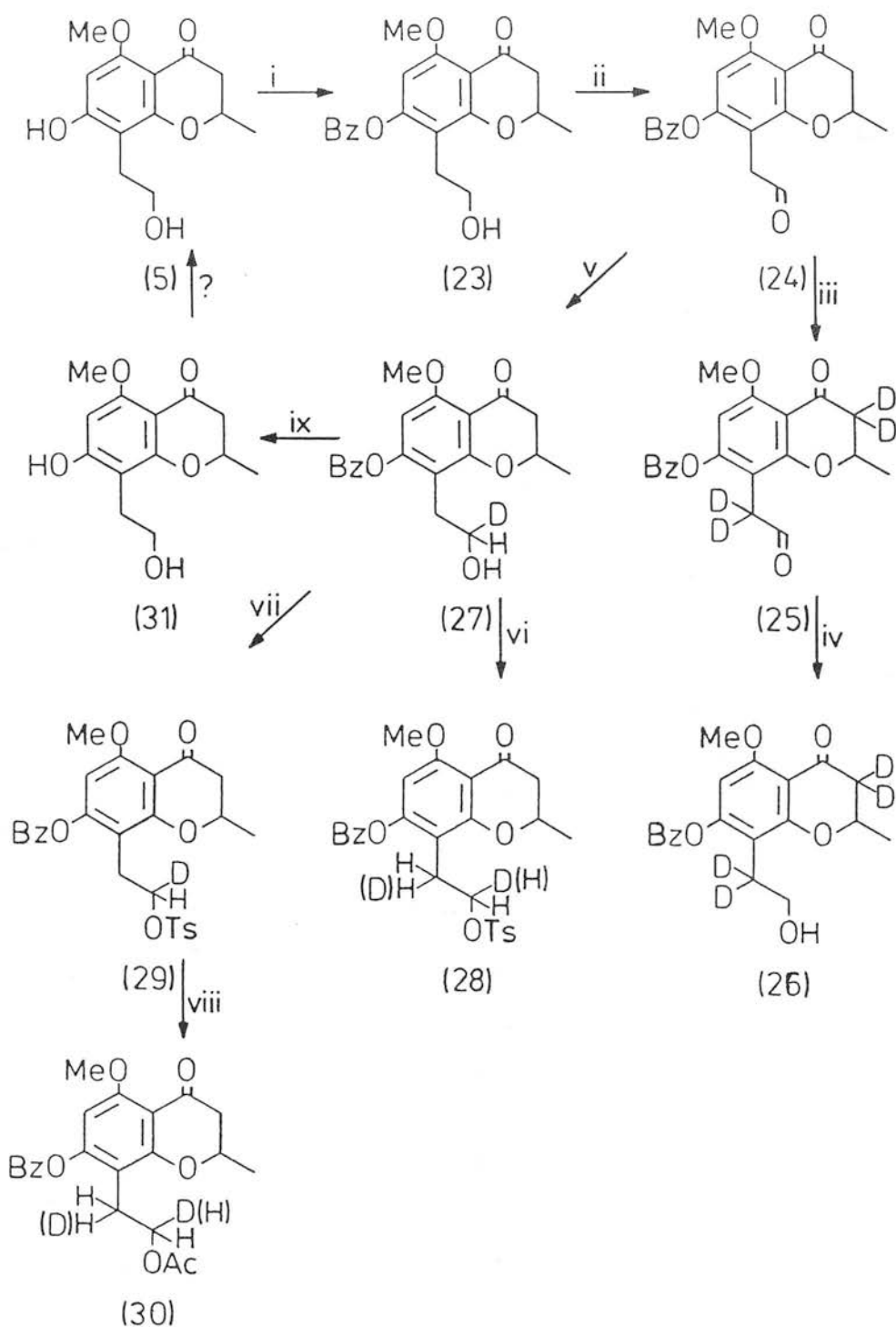
### 2.2.3. Malonate Incorporation into LL-D253 $\alpha$

In a preliminary experiment, [2- $^{14}\text{C}$ ]-malonate was administered to *P. pigmentivora*, and the LL-D253 $\alpha$  obtained was purified and recrystallised to constant activity. The dilution of label of 3.4, or about 20 per labelled site, suggested that [ $^{13}\text{C}$ ]-malonate would be incorporated efficiently enough to be detectable by  $^{13}\text{C}$  n.m.r.. As diethyl [2- $^{13}\text{C}$ ]-malonate was not routinely available, it had to be synthesised. In 1950 Ropp synthesised ethyl [2- $^{14}\text{C}$ ]-acetate by heating sodium [2- $^{14}\text{C}$ ]-acetate with triethyl phosphate (scheme 7)<sup>21</sup>. In 1981, Leete reported the formation of the lithium enolate (21) of ethyl [2- $^{13}\text{C}$ ]-acetate (20) at low temperatures, and reacted it with ethyl chloroformate to give diethyl [2- $^{13}\text{C}$ ]-malonate (22)<sup>22</sup>. The above procedures were adapted to synthesise ethyl [2- $^{13}\text{C}$ ]-malonate from sodium [2- $^{13}\text{C}$ ]-acetate (scheme 7).

This material was diluted with unlabelled diethyl malonate and administered to *P. pigmentivora*. LL-D253 $\alpha$  was subsequently isolated, and converted to its diacetate. Its n.m.r. spectrum was obtained in the presence of the paramagnetic relaxation agent trisacetylacetonatochromium, in order to normalise the signal intensities; inverse-gated decoupling was used to suppress nuclear Overhauser enhancement (n.O.e.) effects. The intensities of each resonance were compared with that obtained from a natural abundance spectrum run under the same conditions. As expected, all positions directly derived from the methylene carbon of malonate showed enrichments above natural abundance (Table 2), of between 1.6 and 1.8, relative to the average enrichment for the carboxyl-derived positions. The 1-methyl signal showed a relative enrichment of 1.4, in agreement with its position in the starter unit as demonstrated by previous results. The 1' and 2' positions showed enrichments of 1.2 and 1.3

respectively. As both of these positions are derived from the same acetate molecule, the enrichment of this pair of atoms is 1.5. This figure is lower than that of the (malonate) methylene-derived positions (3, 4a, 6 and 8), and is higher than the (malonate) carboxyl-derived positions (2, 4, 5, 7 and 8a). It is however similar in value to that of the (1-) methyl starter unit. While this result should be interpreted cautiously, it is tentatively suggested that the hydroxyethyl side-chain of LL-D253 $\alpha$  and related metabolites is derived from reduction of an acetate side-chain, rather than from degradation of an acetoacetate side-chain.





Scheme 8: Synthetic routes to deuterated LL-D253α derivatives.

Reagents: i)  $\text{PhCH}_2\text{Br}$ ,  $\text{K}_2\text{CO}_3$ ; ii) PCC; iii)  $\text{CH}_3\text{ONa}$ ,  $\text{CH}_3\text{OD}$ ; iv)  $\text{NaBH}_4$ ; v)  $\text{NaBD}_4$ ; vi)  $\text{TsOH}$ ; vii)  $\text{TsCl}$ , pyridine; viii)  $\text{AcOH}$ ; ix)  $\text{Pd/C}$ .

#### 2.2.4. Synthesis and Rearrangement of Specifically Deuteriated LL-D253 $\alpha$

##### Derivatives

It was decided to synthesise specifically labelled derivatives of LL-D253 $\alpha$ , and subject them to various acidic and basic conditions, in order to determine how readily randomisation of this label would occur. The label chosen was deuterium for its ease of introduction and ready detectability.

The synthetic route is outlined in scheme 8. The benzyl ether (23) was prepared from LL-D253 $\alpha$  by standard methodology. It was then carefully oxidised to (24) using pyridinium chlorochromate, known to favour formation of aldehydes, rather than carboxylic acids, from primary alcohols. The aldehyde was immediately subjected to exchange conditions (sodium methoxide in deuteriomethanol solution). The proton n.m.r. signals due to the 1' and 3 positions diminished within three minutes, and the 2' aldehyde triplet was replaced by a singlet; thus exchange was rapid under these conditions. The product (25), known to be unstable with respect to air oxidation<sup>4</sup>, was immediately reduced using sodium borohydride. This too was a rapid reaction and was carefully monitored by analytical thin layer chromatography to minimise overreduction at the ketonic carbon. The resulting product (26), once purified, yielded an identical proton n.m.r. to that of the starting alcohol (23), except that the 1'- and 3- signals had almost disappeared, the 2'- signal was predominantly a singlet, and the 2 signal had sharpened to a quartet. Deuterium n.m.r. of (26) gave two signals at 2.57 and 2.90 p.p.m., corresponding to the 3 and 1' positions respectively. The mass spectrum of (26) gave major peaks three and four atomic mass units higher than that of the parent peak of (23).

It was decided as an alternative method to directly reduce (24) using sodium borodeuteride. This was carried out successfully to give (27) whose proton n.m.r. was identical to that of (23), except that the 1' signal was now a

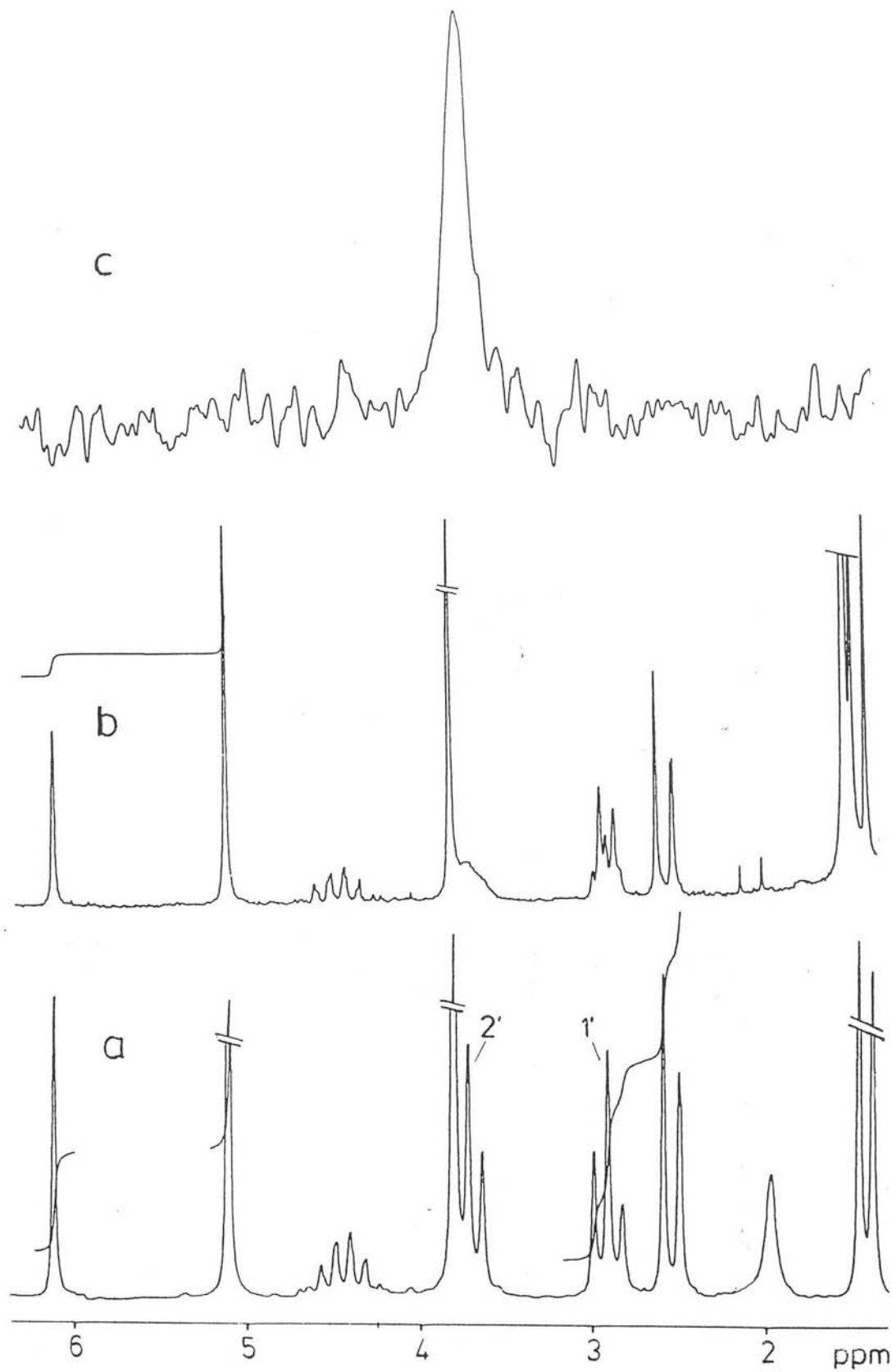


Figure 2. Nmr. spectra of  $[2'-^2\text{H}]\text{-LL-D253}\alpha\text{-7-O-benzyl ether}$ .

2a: 80MHz.  $^1\text{H}$  spectrum of unlabelled material.

2b: 80MHz.  $^1\text{H}$  spectrum.

2c: 30.7MHz.  $^2\text{H}$  spectrum.

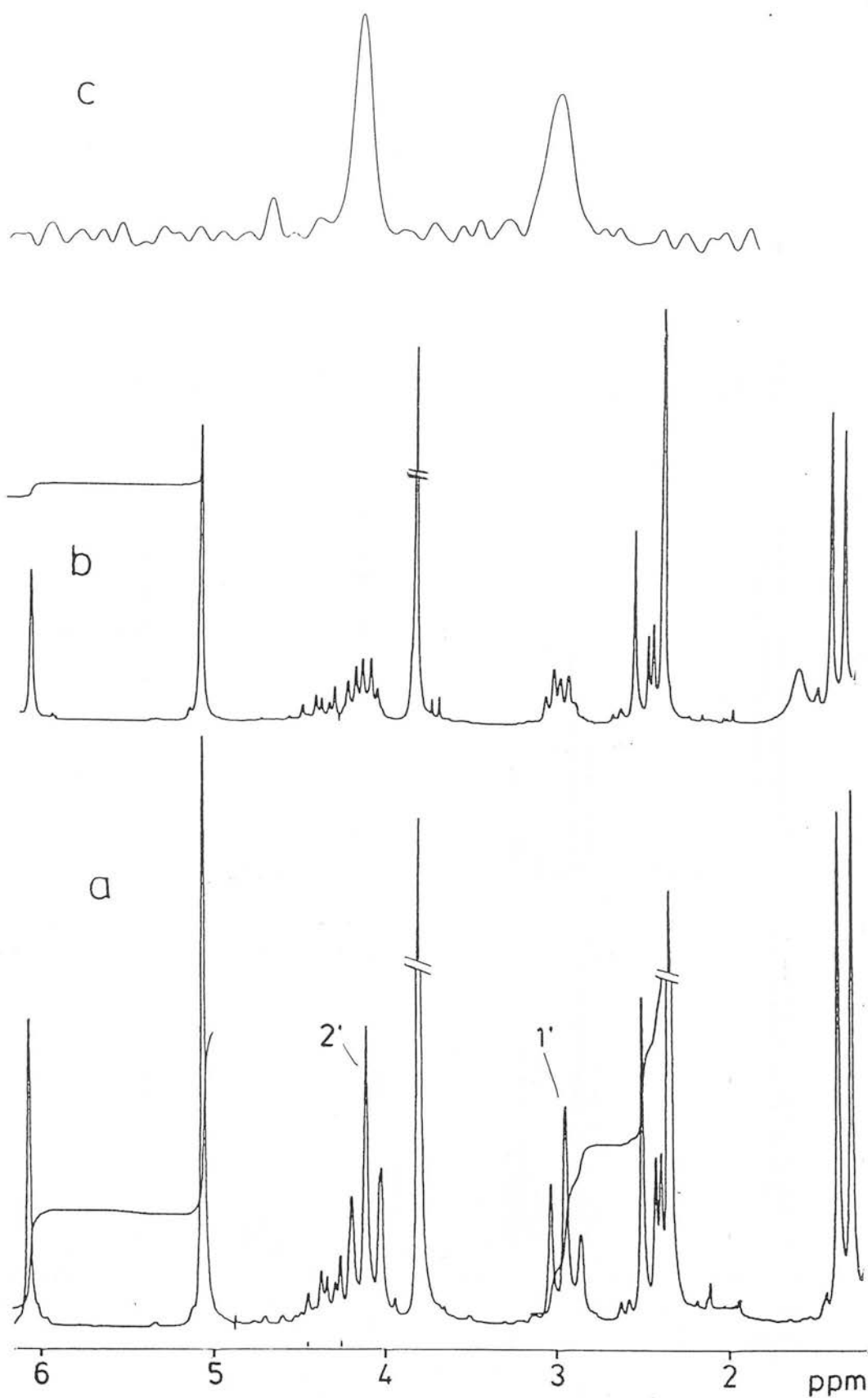


Figure 3. Nmr. spectra of [1',2'- $^2\text{H}$ ]-LL-D253 $\alpha$ -7-O-benzyl ether-2'-tosylate.  
 3a: 80MHz.  $^1\text{H}$  spectrum of unlabelled material.  
 3b: 80MHz.  $^1\text{H}$  spectrum.  
 3c: 30.7MHz.  $^2\text{H}$  spectrum.

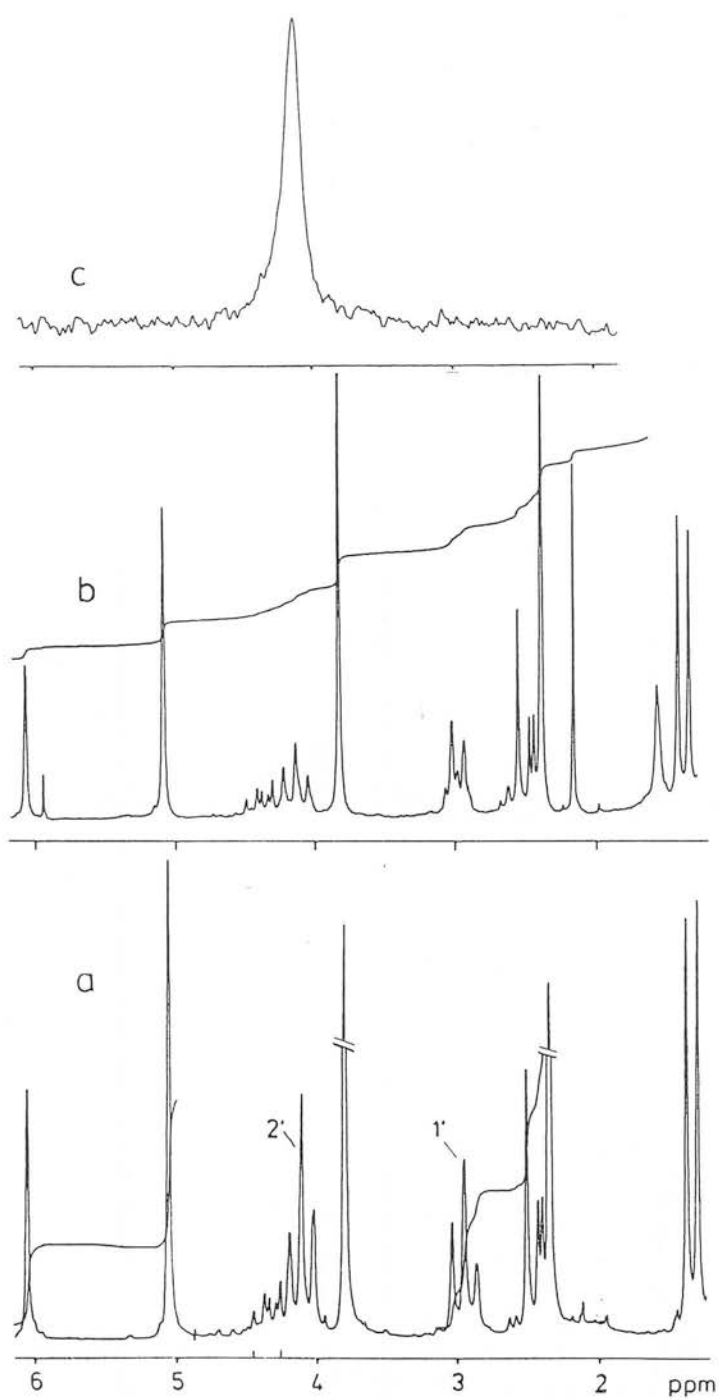


Figure 4. Nmr. spectra of  $[2'-^2\text{H}]\text{-LL-D253}\alpha\text{-7-O-benzyl ether-2'-tosylate}$ .  
4a: 80MHz.  $^1\text{H}$  spectrum of unlabelled material.  
4b: 80MHz.  $^1\text{H}$  spectrum.  
4c: 55.3MHz.  $^2\text{H}$  spectrum.

doublet superimposed on a triplet, and the 2' signal had diminished in intensity (Figure 2). The deuterium n.m.r. of (27) showed a signal at 3.73 ppm (Figure 2), corresponding to the 2' position of (23). Mass spectrometry gave a parent peak one mass unit higher than that of (23).

The toluenesulphonate ester (28) was prepared from (27) by refluxing it with a slight molar excess of p-toluenesulphonic acid in benzene. Its proton n.m.r. spectrum (Figure 3) showed superimposed doublets and triplets for both 1' and 2' positions, indicating randomisation of the two side-chain carbon atoms had occurred. This was confirmed by deuterium n.m.r.; two signals at 2.97 and 4.13 p.p.m. were observed. It is interesting to note that there appeared to be a slight excess of the unrearranged product.

The toluenesulphonate ester was also prepared under basic conditions (toluenesulphonyl chloride, pyridine) to give (29); its proton n.m.r. spectrum (Figure 4) gave a diminished triplet corresponding to the 2' position, and a superimposed triplet and doublet for the 1' position. The deuterium n.m.r. spectrum gave one signal only, at 4.13 p.p.m.; thus basic conditions did not lead to randomisation of label.

This toluenesulphonate ester (29) was subjected to acetolysis conditions. The resulting acetate (30) showed two singlets in its deuterium spectrum (Figure 5). Again there seemed to be a slight excess of unrearranged acetate.

Thus it has been demonstrated that a close analogue of LL-D253 $\alpha$  can undergo an acid-catalysed randomisation at the side-chain. In order to gauge whether such a rearrangement could occur *in vivo*, the labelled natural product [2-<sup>2</sup>H]-LL-D253 $\alpha$  was prepared and subjected to growth conditions of *P. pigmentivora*. The deuterium-labelled benzyl ether (27) was hydrogenolysed without rearrangement to give the 2'-deuteriated natural product (31). This was

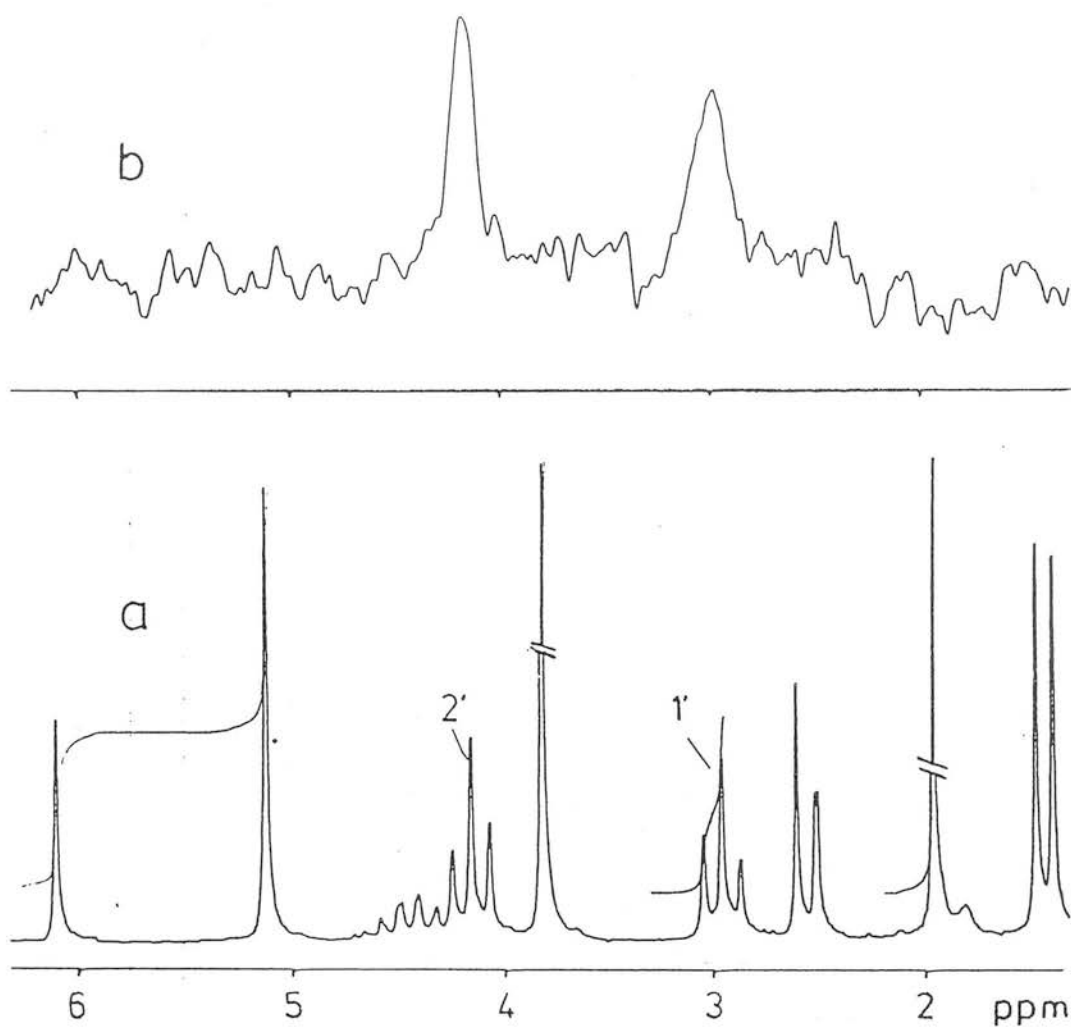
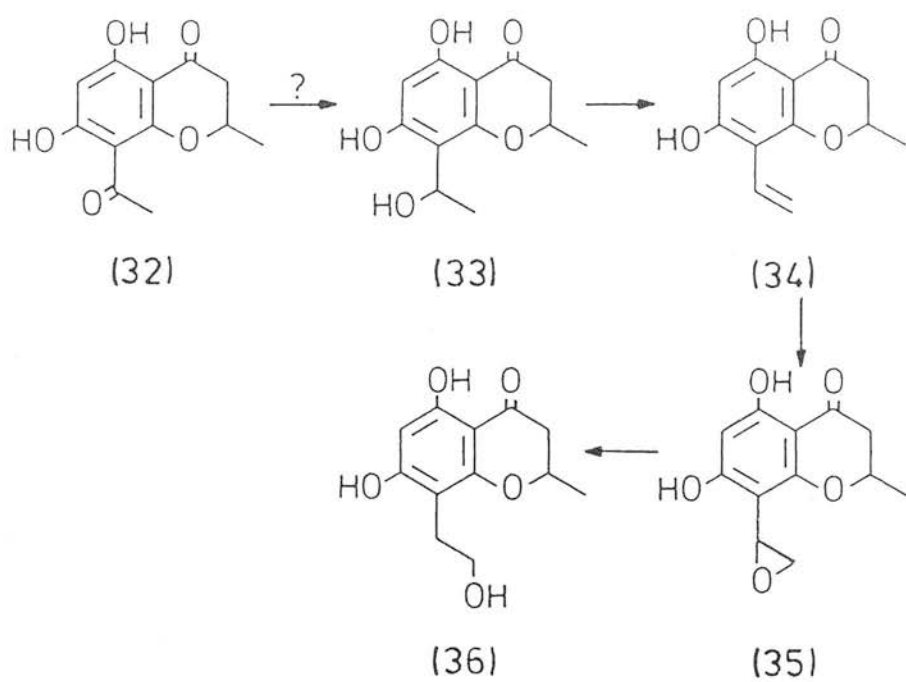


Figure 5. Nmr. spectra of  $[1',2'\text{-}^2\text{H}]\text{-LL-D253}\alpha\text{-7-O-benzyl ether-2'-acetate}$ .  
5a: 80MHz.  $^1\text{H}$  spectrum of unlabelled material.  
5b: 55.3MHz.  $^2\text{H}$  spectrum.

administered to a growing culture of *P. pigmentivora*, 60 hours after inoculation, and reisolated a further 24 hours later. As expected, the deuterium label had been diluted, but all label remained at the 2'-position, as determined by deuterium n.m.r. spectroscopy. It is possible that the administered compound did not gain access to the site of metabolite production, due to permeability problems. However it can be concluded that the randomisation of label is not an artifact of the mildly acidic isolation process.





Scheme 9

### 2.2.5. Synthesis of Putative LL-D253 $\alpha$ Precursors

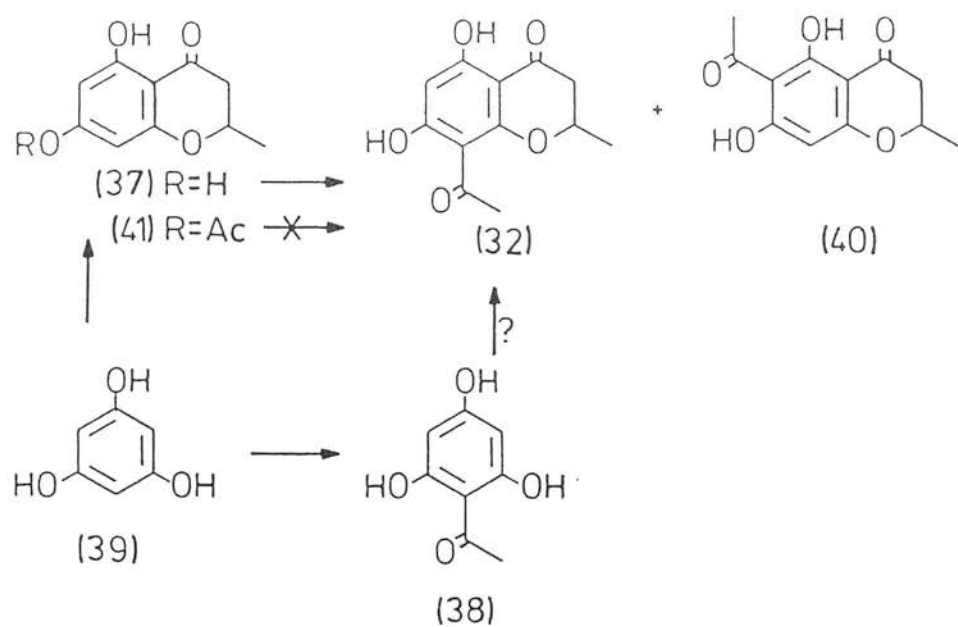
It was proposed to synthesise putative precursors (10), (11), (12) and (13) for both R=H and R=Methyl.

One obvious initial target compound was the acetylchromanone (32). However it was expected that selective reduction of the 1' carbonyl group, in the presence of the 3- carbonyl, would be a major problem. For this reason the hydroxyethylchromanone (33) would have to be gained by another route; once obtained, it was reasoned that the other desired compounds would be accessible from this, by dehydration to the alkene (34), then epoxidation to (35), followed by reductive ring-opening of the epoxide to give the desired hydroxyethyl compound (36) (scheme 9).

Two major problems were anticipated in the synthesis of these apparently simple compounds:

1. The necessity for the correct regiochemistry, ie an 8-substituted, not a 6-substituted chromanone. If possible it was hoped to avoid a mixture of these two isomers.
2. With an eventual view to a labelled synthesis, the most efficient "cold" synthesis may not be the best labelled synthesis. In general it is best to introduce the label as late as possible in the synthesis, and to ensure that subsequent steps are high yielding.

As stated in the previous chapter, most routes to chromanones utilise the Friedel-Crafts reaction. Thus the obvious starting materials to (32) are



Scheme 10

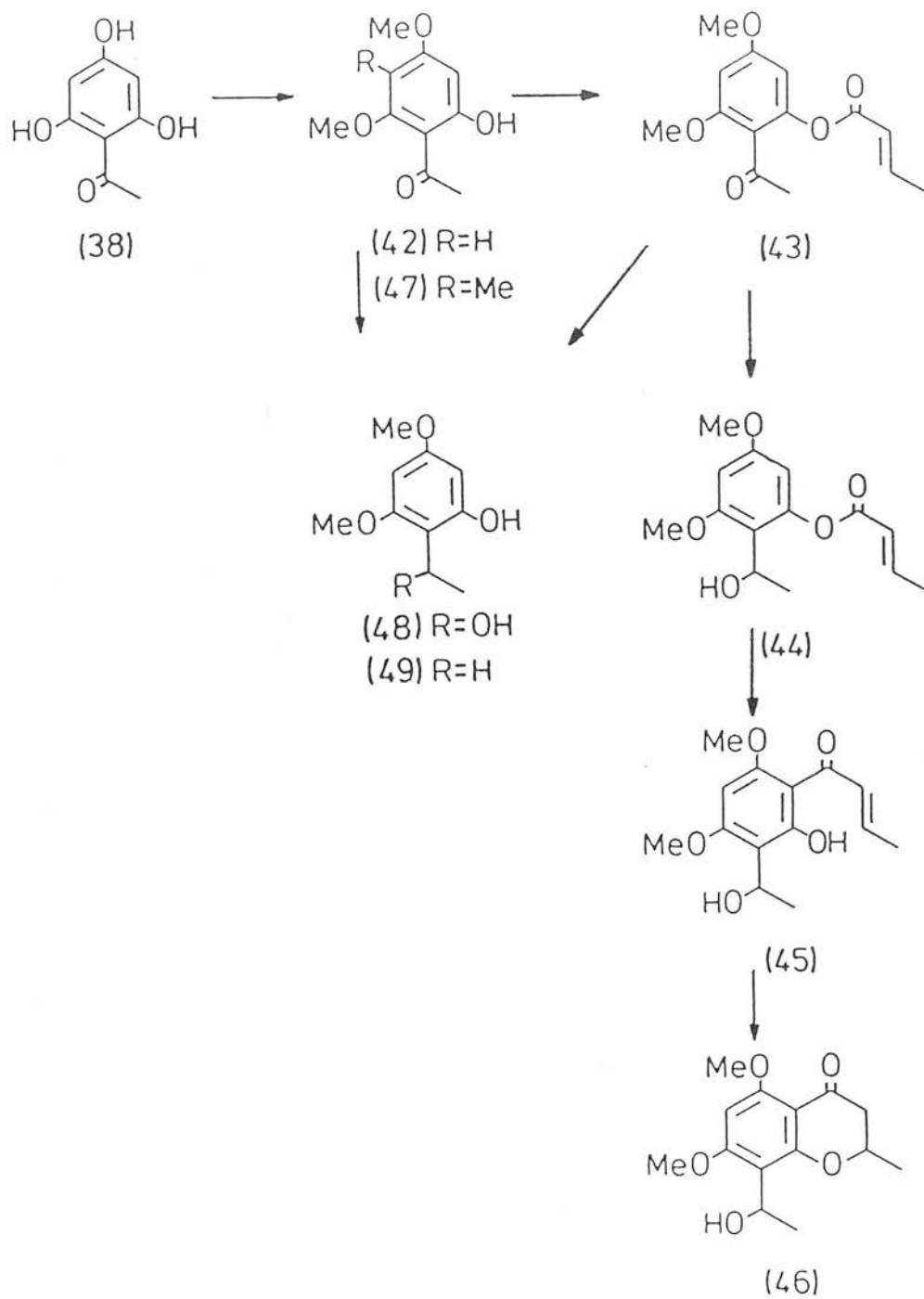
5,7-dihydroxy-2-methyl-4-chromanone (37) or phloracetophenone (38), both of which have been prepared from phloroglucinol (39) (scheme 10).

The synthesis of (37) from phloroglucinol and crotonic anhydride<sup>23</sup>, using aluminium trichloride as catalyst, and nitrobenzene as solvent has been reported. This method gives low yields, and the work-up is tedious and messy. Boron trifluoride has been used as catalyst in the synthesis of 5,7-dihydroxy-2,2-dimethyl-4-chromanone from dimethylacrylic acid and phloroglucinol<sup>24</sup>. This procedure was repeated for crotonic acid and phloroglucinol; the result was a slight improvement in yield of (37), but with the same problems of product isolation.

A new route to 2,2-dimethyl chromanones was recently reported<sup>25</sup>; it uses methanesulphonic acid as catalyst, and phosphorus pentoxide. The use of this powerful dehydrating agent means that the appropriate unsaturated acid may be used directly, and the chromanone ring-closure step proceeds spontaneously. This method was applied to the synthesis of (37) with an improved yield and easier isolation. However a tricyclic product, formed from a *bis*-crotonyl adduct, may have decreased the yield. This possibility will be discussed in detail later.

The "thermal reaction" between dimethylacrylic acid and phloroglucinol has been reported<sup>26</sup> to give 5,7-dihydroxy-2,2-dimethyl-4-chromanone, in low yield. This method was attempted for crotonic acid and phloroglucinol, but without success. Also, the attempted condensation of crotononitrile with phloroglucinol, in the presence of the catalyst benzyl trimethylammonium hydroxide<sup>27</sup>, returned only starting material.

Acetylation of chromanone (37) using standard conditions gave a 1:1 mixture of two crystalline compounds which had slightly different thin layer



Scheme 11

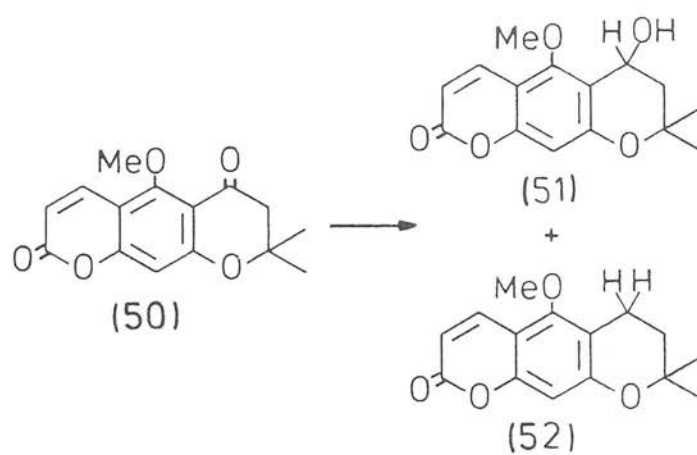
chromatography characteristics, and different, but not diagnostic, n.m.r. signals. These were the isomeric 8-acetyl (32) and 6-acetyl (40) chromanones.

It was hoped that the Fries rearrangement of 7-acetoxy-5-hydroxy-2-methyl-4-chromanone (41) would give predominantly the desired 8-acetyl isomer (32). Accordingly, selective acetylation of (37) (taking advantage of the lower reactivity of the chelated 5-hydroxyl group) gave the desired O-acetate (41). However attempted Fries rearrangement using aluminium chloride catalyst gave the deacetylated compound (37) as the only identifiable product. Attempted rearrangement using "Photo-Fries"<sup>28</sup> conditions returned only starting material. These results are consistent with a report that esters with an "ortho or para carbonyl" functionality are known to respectively "hinder or prevent" the Fries rearrangement<sup>29</sup>. A recent publication has suggested that conversion of the ketone into its cyclic ketal may reduce or negate this effect<sup>30</sup>.

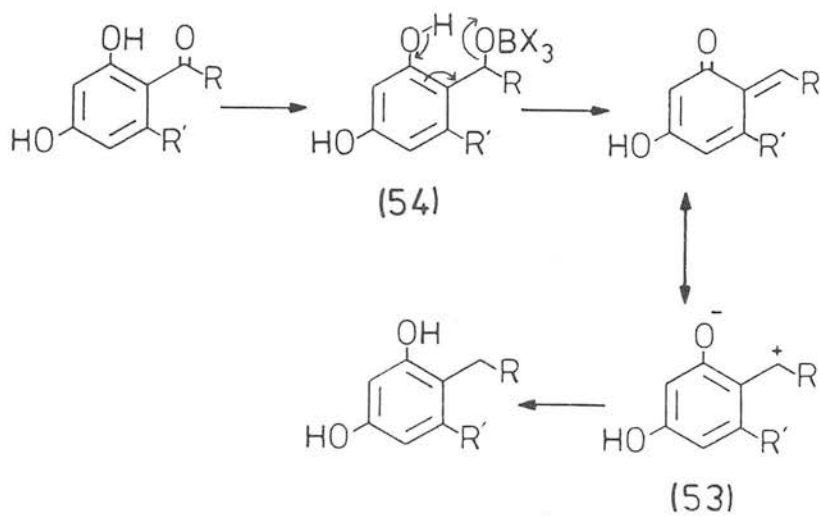
Phloracetophenone (38) was synthesised in high yield from phloroglucinol and acetonitrile by the Hoesch reaction<sup>31</sup>. Attempted condensation of (38) with crotonic acid gave a low-yielding complex mixture.

A possible route to hydroxyethyl chromanones was envisaged (scheme 11). Selective protection of two of the hydroxy groups of (38) would give (42). This could then be converted to its crotonate ester (43), which in turn could be reduced to (44). If this hydroxyethyl ester undergoes a Fries rearrangement it would give (45), which could be cyclised to (46).

To obtain the dimethyl ether (42) of phloracetophenone, iodomethane and potassium carbonate were initially used. This however gave a mixture of the desired compound and its 5-methyl derivative (47)<sup>32</sup>, the latter from C-methylation due to the highly nucleophilic nature of the phloracetophenone nucleus in basic conditions. The products were very difficult to separate.



Scheme 12



Scheme 13

Fortunately, an alternative route, using dimethyl sulphate, was available<sup>33</sup>; this afforded the desired compound cleanly, in good yield.

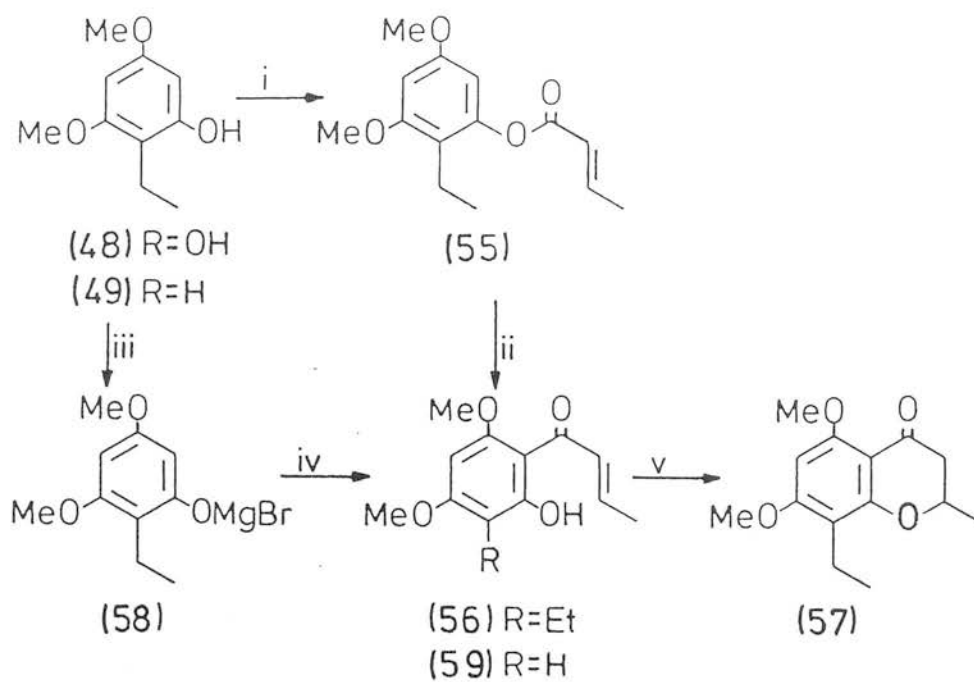
In order to synthesise the crotonate ester (43), 2-hydroxy-4,6-dimethoxyacetophenone and crotonyl chloride were refluxed in benzene with magnesium ribbon<sup>34</sup>. However mainly starting material was returned. The use of higher-boiling toluene instead of benzene gave the desired ester (43), a highly crystalline solid.

The reduction of this compound to the corresponding hydroxyethyl ester (44) was attempted. All efforts using sodium borohydride resulted in hydrolysis of the crotonyl moiety. The use of sodium borohydride adsorbed on silica gave 2-hydroxy-4,6-dimethoxyacetophenone as the first isolable product. When reduction of the acetyl side-chain did occur, the result was a mixture of the hydroxyethylphenol (48) and ethylphenol (49), which were difficult to separate.

It was then reasoned that if (48) could be made cleanly, it could be converted to its crotonate ester which could in turn be subjected to Fries rearrangement conditions. However all attempts at reduction using sodium borohydride gave a mixture of the ethyl and hydroxyethyl compounds. Reduction using sodium borohydride on silica or sodium cyanoborohydride were slower, but no more selective.

This overreduction of  $\alpha$ -aryl ketones to arylalkanes has been reported briefly in the literature; In the structural determination of clausenin, The reduction of its methyl ether (50) using sodium borohydride gave a mixture of hydroxy (51) and methylene (52) products (scheme 12)<sup>35</sup>. In 1969 the borohydride reduction of a series of aryl alkyl ketones was reported. The proportion of fully reduced (methylene) to hydroxy compound increased with the number of ortho and/or para hydroxy or methoxy substituents on the





Scheme 14: Synthetic routes to ethylchromanones.  
 Reagents: i)  $\text{CH}_3\text{CH}:\text{CHCOCl}$ , Mg, PhMe; ii)  $\text{AlCl}_3$ ,  $150^\circ\text{C}$ ;  
 iii)  $\text{EtMgBr}$ ; iv)  $\text{CH}_3\text{CH}:\text{CHCOCl}$ , PhMe; v) aqueous NaOH.

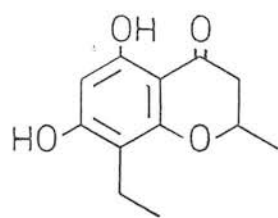
aromatic ring. This has been rationalised by the increased electron-donating ability of the aryl nucleus, which can stabilise a secondary carbocation such as (53), formed by expulsion of an oxygen-boron species, from a postulated intermediate such as (54) resulting from carbonyl reduction (scheme 13)<sup>36</sup>.

Attempted catalytic reduction of (42) in ethanol or glacial acetic acid only returned starting material; refluxing with sodium in ethanol<sup>37</sup> had the same result.

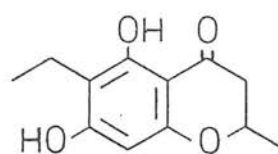
However the ethyl compound (49) itself is useful, as the ethyl chromanone (8) is also a *Phoma* metabolite. In addition it might be possible to functionalise the benzylic methylene, by bromination followed by hydrolysis. Thus 2-hydroxy-4,6-dimethoxyacetophenone was reduced with excess sodium borohydride. The desired compound was obtained, but in low yield. For this reason the Clemmensen reduction<sup>38,39</sup> (zinc amalgam-hydrochloric acid) was used. The product was a gum, but after removing water by azeotroping with benzene, an off-white solid (49) was obtained. A recent publication has reported the use of sodium cyanoborohydride for this reaction<sup>40</sup>.

The crotonate ester (55) was obtained in the manner described for the acetyl analogue. This was subjected to standard Fries rearrangement conditions<sup>41</sup>, and gave the desired product (56), in low yield; this was identical by analytical thin layer chromatography to the product described in the following paragraph (scheme 14).

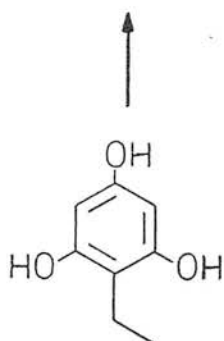
The use of the "magnesium-directed" Friedel-Crafts reaction should result in acylation of phenol (49) ortho to the free hydroxyl group<sup>42</sup>, thereby achieving the desired regioselectivity of substitution leading to an *8*-ethyl-chromanone (57). Thus the magnesium phenolate (58) was made, and reacted with crotonyl chloride under anhydrous conditions. The desired crotonylphenol (56) was



(60)



(62)



(61)

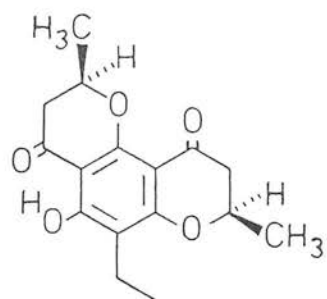
Scheme 15

obtained, along with a small amount of the chromanone (57). The former exhibited similar spectral properties to those of its desethyl analogue (59), a metabolite of the herb *Dysophilla stellata*<sup>43</sup>, and of *D. tometosa* (see Chapter 1.2.4)<sup>44</sup>. The latter compound (59) was later synthesised from 2-hydroxy-4,6-dimethoxyacetophenone (38) (see Chapter 1.3; scheme 8).

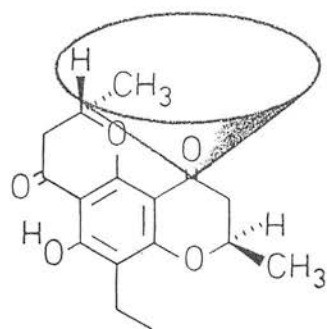
The crotonylphenol was isolated, and was converted into the desired chromanone (57) on stirring with dilute sodium hydroxide. In one experiment, (56) was stirred in sodium deuterioxide and deuteriochloroform; the proton nmr signals corresponding to (56) diminished, to be replaced by signals corresponding to the chromanone.

With a view to synthesising 7-hydroxy-5-methoxy<sup>ox</sup> chromanones, as present in structures (5)-(8), the above procedures leading to 5,7-dimethoxy chromanones are unsatisfactory in that selective demethylation would lead to the isomeric 5-hydroxy-7-methoxychromanone. Thus if a substituted 5,7-dihydroxychromanone were available, the 7-hydroxy moiety could be selectively protected (by, say, benzylation), then 5-O-methylation, followed by deprotection could be achieved.

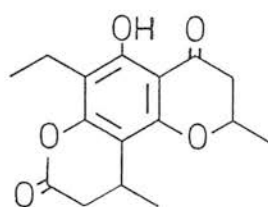
The preparation of 5,7-dihydroxy-8-ethyl-2-methyl-4-chromanone (60) by condensation of C-ethylphloroglucinol (61) with crotonic acid, was investigated. C-ethylphloroglucinol was prepared by Clemmensen reduction of phloracetophenone (scheme 15) but on condensation with crotonic acid in methanesulphonic acid in the presence of phosphorus pentoxide four compounds with similar analytical thin layer chromatography characteristics were obtained. Two compounds with similar nmr. spectra and both of molecular weight 222 a.m.u. were separated with difficulty. They are presumably the desired compound (60) and its regioisomer (62); the data



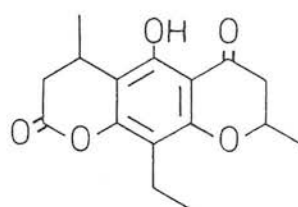
(63)



(64)



(65)

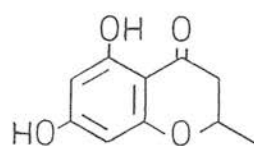


(66)

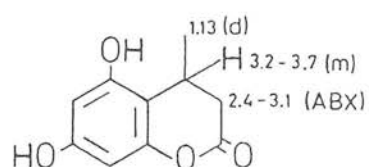
obtained for these compounds is consistent with their proposed structures. However it has not been possible to distinguish between the two isomers; this could readily be achieved by n.m.r. decoupling experiments. Alternatively, the dimethyl ether of one of these compounds should be identical with the 8-ethyl chromanone (57) derived from a different route.

The other two compounds had molecular weights of 290 a.m.u.; Again they had almost identical nmr. spectra, and contained the same signals as the spectra of (60) and (62), minus the aromatic proton and unchelated hydroxyl signals, indicating that an additional ring had been formed. Further signals at 1.17 (doublet), 2.75 (multiplet) and 3.50 (multiplet) were observed. These were very similar in appearance to the chromanone signals at 1.54, 2.75 and 4.70 ppm., and suggested the presence of an extra, but more shielded, fused pyranone ring. Thus structures (63) and (64) were initially proposed for the two compounds. The greater shielding of this second ring was attributed to its position in relation to the carbonyl moiety of the adjacent chromanone ring. However the extent of the shielding (1.0 and 0.3 ppm. for the quartet and doublet respectively) seemed too high to allow such a rationalisation. Also the coupling constants in this second ring were significantly lower than those observed in the chromanone rings of (60) and (61). A second, though initially less obvious, explanation is that these signals are due to the presence of a  $\alpha$ -pyranone (dihydrocoumarin) ring, for example, structures (65) and (66), rather than an additional  $\gamma$ -pyranone (or chromanone) ring. Inspection of the literature would appear to support this; for compounds with a similar structure, the nmr. data quoted often covers large chemical shift ranges, is incompletely assigned, and no coupling constants are available. Nevertheless this data shows a good correlation with the signals reported above of 1.17, 2.75 and 3.50 ppm.

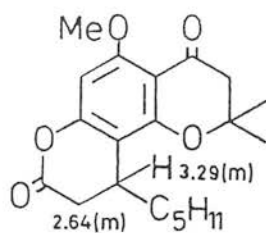
However infra-red data provides more convincing evidence for the presence



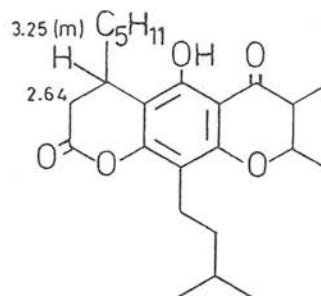
(37)



(67)



(68)



(69)

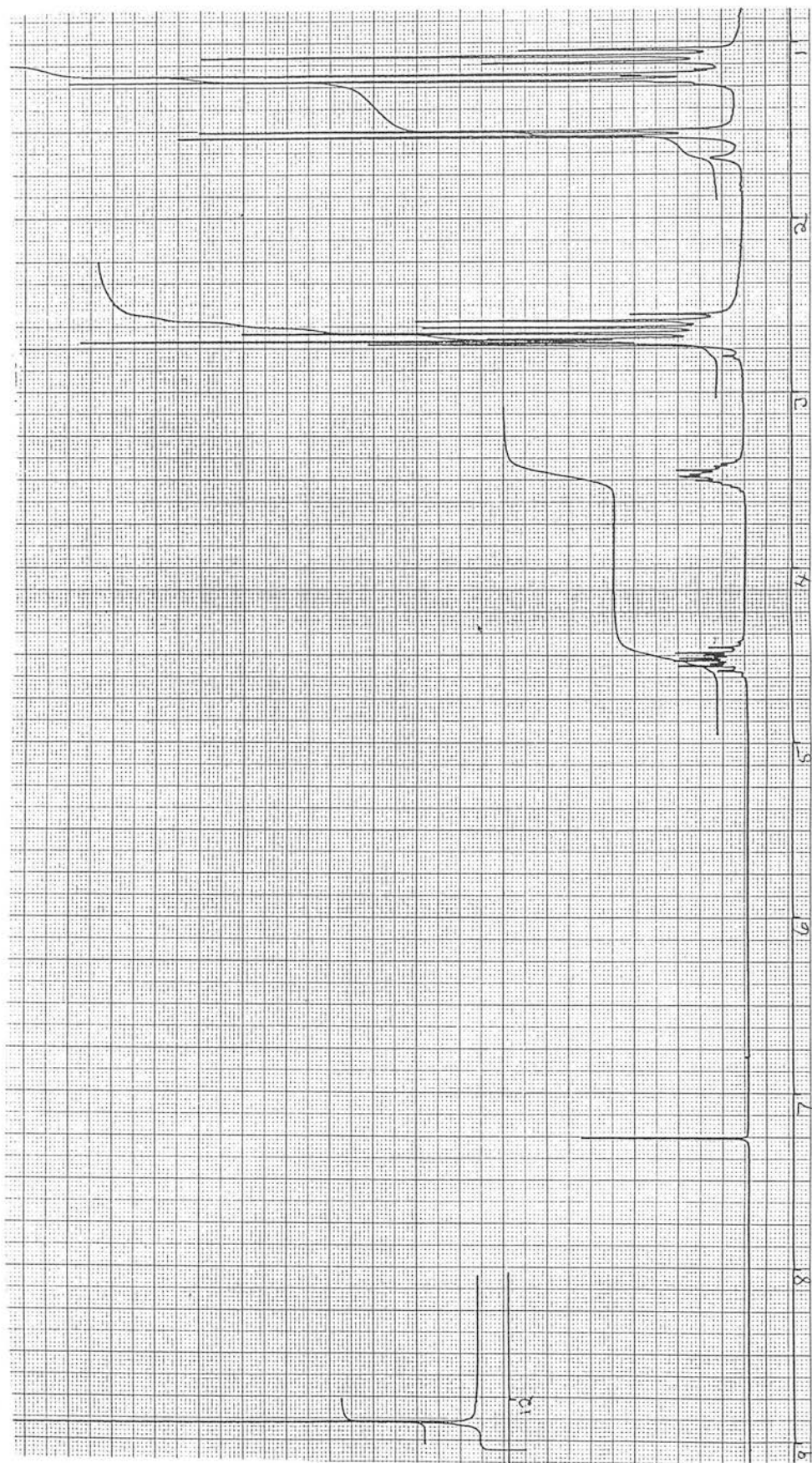


Figure 6: 200 MHz.  $^1\text{H}$  N.M.R. spectrum of "compound B" (see facing page).



of a dihydrocoumarin ring. 5,7-Dihydroxy-2-methyl-4-chromanone (37) and its 6/8-ethyl homologue exhibit carbonyl absorptions at 1630 and 1631  $\text{cm}^{-1}$  respectively. 5,7-Dihydroxy-4-methyl-dihydrocoumarin (67), and compounds (68) and (69) exhibit absorptions at 1760, 1776 and 1786 respectively, clearly due to the presence of the lactone moiety. The carbonyl absorptions for the two compounds of molecular weight 290 are 1770 and 1790  $\text{cm}^{-1}$ . Thus structures (65) and (66) appear to be consistent with all of the experimental data available.

The formation of the compounds proposed above must be rationalised. In such reactions, of either phloroglucinol or its ethyl homologue respectively, no traces of 5,7-dihydroxy-4-methyl-dihydrocoumarin (67) or an ethyl analogue were apparent. Thus it would seem that in both cases, C-acylation occurs to give, after cyclisation, the desired chromanones. The acylation products, due to the presence of an electron-withdrawing group  $\alpha$ - to the aromatic ring, would be expected to render these compounds less nucleophilic than the parent phloroglucinols. Thus it is possible that competition between C- and O-acylation now favours the latter.

Closer scrutiny of the high-field spectra of these two compounds show that certain signals appear doubled up. For the compound with the higher chromatographic mobility of the two ("Compound A"), the coumarin methyl doublet at 1.17 and the hydroxyl signal at 12.08 are split, by 0.012 and 0.027 ppm, respectively. "Compound B", that with a slightly higher polarity, shows doubling of the side-chain methyl triplet at 1.07 and the chromanone methyl doublet at 1.51 ppm, of 0.0036 and 0.0025 ppm respectively (see Figure 6).

It is possible that both compounds "A" and "B" described above are each a mixture of diastereoisomers. A less likely explanation for this phenomenon is

restricted rotation. Variable temperature nmr. studies with compound "B" were conducted; heating to 75°C caused line-broadening, but without any evidence of coalescence.

## 2.3. Experimental

### General Procedures and Instrumentation

Melting points were obtained on a Reichert hot stage machine and are uncorrected. Microanalyses were obtained on a Carlo Erba 1106 elemental analyser. Ultra-violet spectra were obtained using a Varian DMS 90 spectrophotometer; baseline correction for solvent absorption was carried out. Spectra were run in analar chloroform unless otherwise stated.  $\log \epsilon$  is quoted below where  $\epsilon$  is in units of  $\text{dm}^3\text{mol}^{-1}\text{cm}^{-1}$ . Infra-red spectra were recorded on a Perkin Elmer 298 spectrometer;  $\lambda_{\text{max}}$  values quoted were referenced against the polystyrene signal at  $1603\text{cm}^{-1}$ , and the letters in parenthesis refer to relative intensities. Proton nmr spectra were obtained using a Varian EM 360 continuous wave machine, and using Bruker WP 80 SY, WP 200 SY, WH 360 and WH 400 Fourier transform instruments. Deuterium nmr spectra were obtained using Bruker WP 80 SY, WP 200 SY and WH 360 instruments.  $^{13}\text{C}$  spectra were obtained using WP 200 SY and WH 400 instruments. Chemical shifts quoted below are relative to tetramethylsilane (tms);  $\delta_{\text{H}}$  and  $\delta_{\text{C}} = 0.0$  ppm. The letters in parenthesis refer to signal multiplicity. Mass spectra were obtained from a Kratos MS 90 instrument; ion formation was achieved by electron impact.

Unless otherwise specified, thin layer chromatography was performed using either analytical (5 x 20 cm.) or preparative (20 x 20 cm.) glass plates coated with a 0.6 mm. layer of silica gel (Fluka AG 60765 Kieselgel GF 254). Unless otherwise specified, chromatograms were visualised by ultra-violet light of 254 nm. wavelength. Reverse-phase high performance liquid chromatography was carried out using a prepacked Versapack C-18  $10\mu\text{m}$  column connected to a Gilson 303 pump with a 803C manometric module and 811 dynamic mixer, and controlled using a data-master disc-drive and Apple microcomputer.

Aqueous solutions for feeding studies were sterilised by autoclaving at 15 p.s.i. for at least 15 minutes.

Where extraction from an aqueous solution into an organic solvent has been reported, the latter was dried using anhydrous magnesium sulphate. When dry solvents are specified, they were prepared by standard procedures<sup>45</sup>. Nitrogen was dried by passing it through a series of traps containing i) concentrated sulphuric acid, ii) glass wool, iii) dilute sodium hydroxide and iv) self-indicating silica gel.

Radiocounting was performed using a Beckman LS 7000 Liquid Scintillation counter, using programme 4 without an automatic quench correction. Counting efficiency was determined by reference to an H-number quench curve. The scintillant used was a solution of butyl-PBD ( $10\text{g l}^{-1}$ ) in methanol-toluene (50:50).

The growth medium used for the production of LL-D253 $\alpha$  contained the following:

ammonium tartrate	0.2% w/v
magnesium sulphate heptahydrate	0.05% w/v
potassium chloride	0.05% w/v
potassium orthophosphate	0.01% w/v
ferrous sulphate heptahydrate	0.001% w/v
glucose	5.0% w/v
corn steep liquor	1.0% w/v
distilled water	to 100% w/v



## Production and Isolation of LL-D253 $\alpha$

*Phoma pigmentivora* (QM 502) was stored at 4°C in darkness under liquid paraffin on slopes of corn-meal agar (Oxoid CM 103). A spore suspension in distilled water was inoculated into 250ml. Erlenmeyer flasks, each containing 75ml. of the growth medium listed above.

This seed culture was incubated for three days at 26°C in constant light on an orbital shaker. The mycelial suspension was used to inoculate further flasks containing the same medium which were in turn incubated under the same conditions for up to twelve days. Most growth occurred within five days by which time the medium had changed from light to dark brown in colour.

The flasks' contents were amalgamated, filtered and washed with water. The combined filtrates were acidified to approximately pH 2 with 2N hydrochloric acid, and extracted into ethyl acetate (4 times), giving a combined volume equal to that of the medium). This was dried and reduced *in vacuo* to give a brown gum. Its constituents were separated by preparative thin layer chromatography, using chloroform-methanol (90:10) as eluant. The band corresponding to an authentic sample of the desired metabolite ( $R_f$  0.5) was isolated. (for larger quantities, initial purification by flash chromatography, using the same eluant, proved more convenient.) Further purification by preparative thin layer chromatography using acetone-chloroform (20:80) as eluant gave the desired compound ( $R_f$  0.3), LL-D253 $\alpha$  (5), as a white solid. Recrystallisation from ethyl acetate-methanol afforded white needles, m.p. 190–192°C. (lit.<sup>1</sup> 188–188.5°C, lit.<sup>2</sup> 191–193°C, lit.<sup>4</sup> 192.5–193°C).

– <sup>1</sup>H N.M.R. (80 MHz., CDCl<sub>3</sub>): 1.44 (3H, d, J=6.3Hz., CHCH<sub>3</sub>), 2.40 (2H, AB of ABX, COCH<sub>2</sub>), 2.90 (2H, t, J=7Hz., ArCH<sub>2</sub>CH<sub>2</sub>), 3.48 (1H, br s, OH), 3.84 (3H, s, OCH<sub>3</sub>), 3.92 (2H, t, J=7Hz.,

ArCH<sub>2</sub>CH<sub>2</sub>), 4.45 (1H, m, J=7Hz., CHCH<sub>3</sub>), 6.13 (1H, s, ArH) ppm.

#### **Incorporation of Sodium [2-<sup>13</sup>C,<sup>2</sup>H<sub>3</sub>]-Acetate into LL-D253α**

A spore suspension of *P.pigmentivora* (QM 502) was inoculated into 250ml. Erlenmeyer flasks, each containing 100ml. of growth medium.

Labelled sodium [2-<sup>13</sup>C,<sup>2</sup>H<sub>3</sub>]-acetate (Amersham; <sup>13</sup>C: 90% enriched, <sup>2</sup>H: 95% enriched) (204mg., 2.37mmoles) and unlabelled sodium acetate (402mg., 4.90mmoles) were mixed and dissolved in distilled water (15ml.) and sterilised.

Sixty hours after inoculation, this solution was distributed among the five flasks. These mycelia grew well, but did not darken as quickly as those in the control flasks. After 132 hours the growth media were filtered and extracted as described previously, to yield a brown oil (537 mg.). This was purified by preparative thin layer chromatography (chloroform-methanol (98:2)), to give a brown solid, slightly impure (by analytical thin layer chromatography) LL-D253α, (158mg.).

#### **Acetylation of LL-D253α Derived from Sodium [2-<sup>13</sup>C,<sup>2</sup>H<sub>2</sub>]-Acetate**

LL-D253α, obtained as described above (158mg., 0.63mmoles), was stirred with acetic anhydride (1ml.) and pyridine (8 drops) for fifteen minutes at 100°C, with the exclusion of moisture. The product was poured onto ice (30ml.), and allowed to reach ambient temperature. This was acidified (2N hydrochloric acid), and extracted into chloroform. The organic layer was washed with water, dried and reduced *in vacuo*. Excess acetic acid was removed *in vacuo* as its azeotrope with carbon tetrachloride. to yield a brown gum (146mg.). This was purified by preparative thin layer chromatography (chloroform-methanol (98:2)),

The main band ( $R_f$  0.8) was isolated as a pale yellow oil which crystallised on standing, *LL-D253 $\alpha$ -diacetate* (109mg., 52%), m.p. 118–121°C, (lit.<sup>1</sup> 120–122°C, lit.<sup>4</sup> 122.5–124.5°C).

– <sup>1</sup>H N.M.R. (60 MHz., CDCl<sub>3</sub>): 1.55 (3H, d,  $J=6\text{Hz.}$ , CHCH<sub>3</sub>), 2.05 (3H, s, CH<sub>2</sub>OCOCH<sub>3</sub>), 2.40 (3H, s, ArOCOCH<sub>3</sub>), 2.70 (2H, d,  $J=7\text{Hz.}$ , COCH<sub>2</sub>), 2.90 (2H, t,  $J=7\text{Hz.}$ , ArCH<sub>2</sub>CH<sub>2</sub>), 3.95 (3H, s, –OCH<sub>3</sub>), 4.25 (2H, t,  $J=7\text{Hz.}$ , ArCH<sub>2</sub>CH<sub>2</sub>), 4.65 (1H, q,  $J=7\text{Hz.}$ , CHCH<sub>3</sub>), 6.35 (1H, s, ArH) ppm.

– <sup>2</sup>H N.M.R. (55 MHz., CHCl<sub>3</sub>): 1.42 (d,  $J=19.5\text{Hz.}$ , 2-<sup>13</sup>CD<sub>n</sub>), 1.45 (s, 2-<sup>12</sup>CD<sub>n</sub>), 2.70 (d,  $J=13.8\text{Hz.}$ , 3-<sup>13</sup>CD), 2.84 (d,  $J=10.8\text{Hz.}$ , Ar<sup>13</sup>CD<sub>n</sub>CH<sub>2</sub>) 4.04 (d,  $J=22.7\text{Hz.}$ , ArCH<sub>2</sub><sup>13</sup>CD<sub>n</sub>) ppm.

– <sup>13</sup>C N.M.R. (100 MHz., CDCl<sub>3</sub>): 20.4 (2-CH<sub>3</sub>), 20.6 (COCH<sub>3</sub>), 20.6 (COCH<sub>3</sub>), 23.1 (C-1'), 45.4 (C-3), 56.0 (OCH<sub>3</sub>), 62.7 (C-2'), 74.1 (C-2), 98.9 (C-6), 109.4 (C-4a), 110.7 (C-8), 155.1 (C-7), 159.9 (C-5), 162.1 (C-8a), 168.6 (COCH<sub>3</sub>), 170.8 (COCH<sub>3</sub>), 190.1 (C-4) ppm.

#### Malonate Incorporation into LL-D253 $\alpha$

##### Incorporation of Diethyl [2-<sup>14</sup>C]-Malonate into LL-D253 $\alpha$

A spore suspension of *P.pigmentivora* (QM 502) was inoculated into ten 250ml. Erlenmeyer flasks each containing 100ml. of growth medium. These flasks were incubated at 26°C in constant light on an orbital shaker.

Diethyl [2-<sup>14</sup>C]-malonate (Amersham; nominally 10 $\mu\text{Ci}$ , in 1ml.) and unlabelled diethyl malonate (500mg., mmoles) were mixed, and this was made

up to 3ml. with ethanol.

A nominally  $0.5\mu\text{Ci}$  sample was analysed by liquid scintillation counting, giving a value of  $0.63\mu\text{Ci}$ ; thus the amount contained in the feed was  $4.03\mu\text{Ci mmol}^{-1}$ .

The above solution was inoculated into five flasks 50 hours after mycelial inoculation. The contents of these flasks grew well, but the mycelia darkened more slowly than those in the control flasks.

After a further 120 hours, the contents of the five flasks were filtered. The amalgamated filtrate was extracted into ethyl acetate (300ml.) which was dried and reduced *in vacuo* to give a yellow gum (520mg.). This was purified by preparative thin layer chromatography (chloroform-methanol (90:10)) to give fairly pure LL-D253 $\alpha$  (70mg.). This was dissolved in analar acetone and a small aliquot containing 1mg. was assayed by liquid scintillation counting. The remainder was further purified by preparative thin layer chromatography (acetone-chloroform (20:80)), to give pure LL-D253 $\alpha$  (35mg.). Again a small aliquot was assayed by liquid scintillation counting. The remainder was recrystallised from methanol and ethyl acetate to small white crystals (20mg.). A portion of this was again assayed by liquid scintillation counting.

### Specimen Calculation

activity of (5) = 8762 cpm/g.

this represents  $2.21 \times 10^6$  cpm/mmol

counting efficiency = 83.4%

thus specific activity of (5) =  $2.65 \times 10^6$  dpm/mmol



thus specific activity of (5) =  $1.19 \mu\text{Ci}/\text{mmole}$

Activity of malonate feed =  $4.03 \mu\text{Ci}/\text{mmol}^{-1}$ .

Activity of impure LL-D253 $\alpha$  =  $2.27 \mu\text{Ci}/\text{mmol}^{-1}$ .

Activity of pure LL-D253 $\alpha$  =  $1.25 \mu\text{Ci}/\text{mmol}^{-1}$ .

Activity of recrystallised LL-D253 $\alpha$  =  $1.19 \mu\text{Ci}/\text{mmol}^{-1}$ .

The dilution of label is given by:

specific activity of metabolite/specific activity of precursor

thus dilution =  $4.03/1.19 = 3.4$ .

Assuming 6 labelled sites per molecule of (5),

dilution = 20.4 per labelled site.

### *Ethyl Acetate*<sup>21</sup>

Anhydrous sodium acetate (812mg., 9.90mmoles) and freshly distilled triethyl phosphate (3.0ml., 3.2g., 17.6mmoles) were refluxed in a small pear-shaped flask filled with glass wool. Precautions were taken to exclude moisture. After one hour, the contents of the flask were allowed to cool, and the apparatus reassembled for distillation. The flask was connected to an oil pump via two traps; the first was maintained at  $-18^{\circ}\text{C}$ , and the second was immersed in liquid nitrogen. The apparatus was heated to  $120^{\circ}\text{C}$  at 5mm.Hg for one hour, then allowed to cool. The second trap contained the desired product, *ethyl acetate*, almost pure by n.m.r., (646mg., 74%).

– I.R. ( $\nu_{\text{max}}^{\text{neat}}$ ): 1740 (s), 1239 (s)  $\text{cm}^{-1}$ .

- $^1\text{H}$  N.M.R.: (60 MHz.,  $(\text{CD}_3)_2\text{CO}$ ) 0.60 (3H, t,  $J=6.5\text{Hz.}$ ,  $\text{CH}_2\text{CH}_3$ ),  
1.38 (3H, s,  $\text{COCH}_3$ ), 3.54 (2H, q,  $\text{CH}_2\text{CH}_3$ ).

### *Ethyl [2- $^{13}\text{C}$ ]-Acetate*

The above procedure was repeated, using sodium [2- $^{13}\text{C}$ ]-acetate (Amersham; 90% enriched)(1.043g., 12.56 mmoles) and triethyl phosphate (3.9ml.,4.2g.,22.9mmoles). The isolated product was a clear liquid, *ethyl [2- $^{13}\text{C}$ ]-acetate*, (955mg.,85% crude), which was kept dry until further use.

### *Diethyl Malonate*<sup>22</sup>

All glassware was dried at  $140^\circ\text{C}$  prior to use; precautions were taken to exclude moisture. Bis-trimethylsilylamine (5.6ml.,4.3g.,26.5mmoles) in dry tetrahydrofuran was cooled to  $-65^\circ\text{C}$ , and *n*-butyllithium (31.6mmoles in hexane) was added. The solution warmed to ambient temperature, and was further cooled to  $-72^\circ\text{C}$ . Dry ethyl acetate (1.45ml.,1.31g.,14.9mmoles) in tetrahydrofuran (3.5ml.) was added over 10 minutes. After one hour, freshly distilled ethyl chloroformate (1.4ml.,1.6g.,14.6mmoles) was added dropwise. The solution was stirred for a further 2.5 hours, and allowed to warm to room temperature.

Hydrochloric acid (6N, 4ml.), then water (20ml.), and ether (100ml.), were added to the product. The aqueous layer was washed with ether (50ml.). This was washed with hydrochloric acid (20ml.), water (50ml.) and sodium bicarbonate solution (5%; 50ml.). The combined ethereal extracts were dried and reduced *in vacuo* to yield a yellow liquid (1.95g., 82%).

- I.R. ( $\nu_{\text{max}}^{\text{neat}}$ ):  $1750\text{ (s) cm}^{-1}$ .

-  $^1\text{H}$  N.M.R.: (60 MHz., neat) 0.92 (6H, t,  $J=7\text{Hz.}$ ,  $\text{CH}_2\text{CH}_3$ ): 3.02 (2H, s,  $\text{CH}_2(\text{CO})_2$ ), 3.87 (4H, q,  $J=7\text{Hz.}$ ,  $\text{CH}_2\text{CH}_3$ ).

### *Diethyl [2- $^{13}\text{C}$ ]-Malonate*

The above procedure was repeated using ethyl [2- $^{13}\text{C}$ ]-acetate (1.193g., 1.07g., 12.1mmoles), bis-trimethylsilylamine (4.7ml., 3.6g., 22.3mmoles), *n*-butyllithium (27.2mmoles in hexane) and ethyl chloroformate (1.3ml., 1.48g., 13.6mmoles). The product was a pale yellow liquid, *diethyl [2- $^{13}\text{C}$ ]-malonate* (1.669g., 86% crude), contaminated by a compound with a chemical shift of -0.25 ppm. Its purity by gas chromatography (10% APL column, 139°C) was 80%, hence net yield = 69%.

### **Incorporation of Diethyl [2- $^{13}\text{C}$ ]-Malonate into LL-D253 $\alpha$**

A spore suspension of *P. pigmentivora* (QM 502) was inoculated into 250ml. Erlenmeyer flasks, each containing 100ml. of medium. These were grown on an orbital shaker at 26°C in constant light.

Labelled diethyl malonate (203mg., 1.26mmoles) was mixed with unlabelled diethyl malonate (406mg., 2.53mmoles), and dissolved in methanol (3ml.). This was distributed among five flasks, 56 hours after inoculation; all mycelia grew as well as those in the control flasks. After five days, the growth media were filtered and extracted as described previously, to give a brown oil (538mg.). Its components were separated by preparative thin layer chromatography (chloroform-methanol (90:10)). A band which had the same  $R_f$  as a spot of standard LL-D253 $\alpha$  was isolated (163mg.). This was further purified by

preparative thin layer chromatography (acetone-chloroform (90:10)) to give a white solid, LL-D253 $\alpha$  (49mg.), m.p. 187–190°C (lit.<sup>1</sup> 188–188.5°C, lit.<sup>2</sup> 191–193°C, lit.<sup>4</sup> 192.5–193°C).

#### Acetylation of LL-D253 $\alpha$ Derived from Diethyl [2-<sup>13</sup>C]-Malonate

LL-D253 $\alpha$ , obtained as described above (49mg., mmoles) was stirred with acetic anhydride (1ml.) and pyridine (5 drops); precautions were taken to exclude moisture. After removal of solvent a brown oil was obtained. This was purified by preparative thin layer chromatography (chloroform-methanol (98:2)) to give a white crystalline solid, LL-D253 $\alpha$ -diacetate (60mg., 91%), m.p. 121–123°C (lit.<sup>1</sup> 121–122°C, lit.<sup>4</sup> 122.5–124.5).

– <sup>1</sup>H N.M.R. (60 MHz., CDCl<sub>3</sub>): 1.60 (3H, d, J=6Hz., CHCH-{3}), 2.15 (3H, s, CH<sub>2</sub>OCOCH<sub>3</sub>), 2.47 (3H, s, ArOCOCH<sub>3</sub>), 2.74 (2H, d, J=7Hz., COCH<sub>2</sub>), 2.94 (2H, t, J=7Hz., ArCH<sub>2</sub>CH<sub>2</sub>), 3.95 (3H, s, -OCH<sub>3</sub>), 4.25 (2H, t, J=7Hz., ArCH<sub>2</sub>CH<sub>2</sub>), 4.67 (1H, q, J=7Hz., CHCH<sub>3</sub>), 6.33 (1H, s, ArH) ppm.

– <sup>13</sup>C N.M.R. (MHz., CDCl<sub>3</sub>): 20.4 (2-CH<sub>3</sub>), 20.6 (COCH<sub>3</sub>), 20.7 (COCH<sub>3</sub>), 23.0 (C-1'), 45.3 (C-3), 56.0 (OCH<sub>3</sub>), 62.6 (C-2'), 74.0 (C-2), 98.8 (C-6), 109.2 (C-4a), 110.5 (C-8), 154.8 (C-7), 159.7 (C-5), 161.9 (C-8a), 168.4 (COCH<sub>3</sub>), 170.7 (COCH<sub>3</sub>), 189.9 (C-4) ppm.

*7-Benzoyloxy-8-(2-hydroxyethyl)-5-methoxy-2-methyl-4-chromanone (23)*

7-Hydroxy-8-(2-hydroxyethyl)-5-methoxy-4-chromanone (LL-D253α) (5) (286mg., 1.13mmoles), anhydrous potassium carbonate (313mg., 2.27mmoles) and benzyl bromide (288ml., 403mg., 2.35mmoles) in dry acetone were stirred mechanically under reflux with the exclusion of moisture. After 16 hours, analytical thin layer chromatography (chloroform-methanol (96:4)) showed that no starting material remained. After cooling, the product, a yellow solution over a white solid, was reduced *in vacuo*. The resulting white solid was taken up in water (100ml.) and extracted into ethyl acetate (3x50 ml. portions). The organic layer was dried and reduced *in vacuo* to a yellow oil.

This was purified by preparative thin layer chromatography using chloroform-methanol (96:4) as eluant. The most intense band ( $R_f$  0.2), giving a strong blue fluorescence under ultraviolet irradiation, and a yellow colour after spraying with ethanol-sulphuric acid (90:10) and heating, was isolated as a colourless gum (215mg., 55%), which crystallised on standing. This was recrystallised from ethyl acetate to give white needles of *7-benzoyloxy-8-(2-hydroxyethyl)-5-methoxy-2-methyl-4-chromanone* (23), m.p.133-135°C (lit<sup>4</sup>135-136.5°C). The following data were identical to those of an authentic sample.

- I.R. ( $\nu_{\text{max}}^{\text{mull}}$ ): 3462 (br, m), 1647 (m), 1596 (s), 1560 (m), 1273 (m), 1119 (s)  $\text{cm}^{-1}$ .

-  $^1\text{H}$  N.M.R. (80 MHz.,  $\text{CDCl}_3$ ): 1.41 (3H, d,  $J=6.3\text{Hz.}$ , C- $\text{CH}_3$ ), 1.96 (1H, brs, -OH), 2.54 (2H, d,  $J=7.2\text{Hz.}$ ,  $\text{COCH}_2$ ), 2.90 (2H, t,  $J=6.5\text{Hz.}$ ,  $\text{ArCH}_2\text{CH}_2-$ ), 3.71 ((2H, t,  $J=6.5\text{Hz.}$ ,  $\text{ArCH}_2\text{CH}_2-$ ), 3.79 (3H, s,  $-\text{OCH}_3$ ), 4.50 (1H, q,  $J=7.1\text{Hz.}$ , 2-H), 5.10 (2H, s,  $\text{PhCH}_2-$ ),

6.11 (1H, s, ArH), 7.33 (5H, s, C<sub>6</sub>H<sub>5</sub>) ppm.

- M.S. (m/z (%)) 342 (m<sup>+</sup>, 10), 311 (17), 227 (33), 168 (19), 150 (49), 91 (100).

*7-Benzoyloxy-5-methoxy-2-methyl-8-(2-oxoethyl)-4-chromanone (24)*

To a stirred solution of 7-Benzoyloxy-8-(2-hydroxyethyl)-5-methoxy-2-methyl-4-chromanone (LL-D253 $\alpha$ -benzyl ether) (23) (249mg., 0.73mmoles) in dry methylene chloride was added recrystallised, dry pyridinium chlorochromate (296mg., 1.37mmoles), with stirring at ambient temperature. The reaction was monitored by analytical thin layer chromatography (chloroform-methanol (96:4)); a spot corresponding to the substrate (R<sub>f</sub> 0.3) decreased in intensity over three hours, accompanied by the appearance of a less polar compound with the same R<sub>f</sub> (0.5) as an authentic sample of the anticipated product.

Water was added, and the product extracted into ether (4x50ml. portions). The ethereal solution was dried and reduced *in vacuo* to yield an off-white solid (218mg., 88%), showing identical analytical thin layer chromatography and n.m.r. characteristics to an authentic sample of *7-benzoyloxy-5-methoxy-2-methyl-8-(2-oxoethyl)-4-chromanone* (24), m.p.119-125°C (lit.<sup>4</sup>123-127°C dec.).

- <sup>1</sup>H N.M.R. (60 MHz., CDCl<sub>3</sub>): 1.45 (3H, d, J=6Hz., C-CH<sub>3</sub>), 2.60 (2H, d, J=7Hz., 3-H<sub>2</sub>), 3.67 (2H, d, J=1.5Hz., ArCH<sub>2</sub>-), 3.87 (3H, s, -OCH<sub>3</sub>), 4.2-4.8 (1H, s, 2-H), 5.15 (2H, s, PhCH<sub>2</sub>-), 6.20 (1H, s, ArH), 7.36 (5H, s, C<sub>6</sub>H<sub>5</sub>), 9.60 (1H, t, J=1.5Hz., -CHO).

*[1,3-<sup>2</sup>H<sub>4</sub>]-7-Benzoyloxy-8-(2-hydroxyethyl)-5-methoxy-2-methyl-4-chromanone (26)*

Crude 7-benzyloxy-5-methoxy-2-methyl-8-(2-oxoethyl)-4-chromanone (24) (188mg., 0.55mmoles) was dissolved with difficulty in deuteriomethanol (0.5ml.) in a 5 mm. n.m.r. tube to give a yellow solution, the 60 MHz. n.m.r. spectrum of which was recorded. Compared with the spectrum run in deuteriochloroform, signals due to the aldehyde proton (9.65 ppm) and the protons on the adjacent carbon (3.60 ppm) had diminished, with the appearance of a doublet at 2.85 ppm, suggesting the presence of an acetal or hemiacetal in methanolic solution. After standing at 24 hours at ambient temperature an n.m.r. spectrum was recorded, with no apparent changes.

A solution of sodium deuteriomethoxide in deuteriomethanol was prepared by adding sodium (25mg., 1.09mmoles) to cooled deuteriomethanol (0.25ml.), and a portion of this (3 drops) was added to the n.m.r. tube, whereby the yellow colour darkened. The n.m.r. spectrum was recorded immediately; signals at 2.45, 2.80, 3.60 and 9.65 ppm had greatly diminished. No further changes took place over the next ten minutes. The product was acidified (2N hydrochloric acid) and extracted into ethyl acetate (2x 50ml.), dried (magnesium sulphate) and reduced *in vacuo* to yield a yellow oil (191mg.). The 60 MHz. n.m.r. spectrum of the crude product indicated an absence of signals at 2.60 and 3.60 ppm; also, the aldehyde signal at 9.60 was now a singlet. Otherwise, the spectrum was identical to that of the starting aldehyde:

- <sup>1</sup>H N.M.R. (60 MHz., CDCl<sub>3</sub>): 1.45 (3H, d, J=6Hz., C-CH<sub>3</sub>), 3.85 (3H, s, -OCH<sub>3</sub>), 4.50 (1H, m, 2-H), 5.15 (2H, s, PhCH<sub>2</sub>-), 6.20 (1H, s, ArH), 7.35 (5H, s, C<sub>6</sub>H<sub>5</sub>), 9.65 (1H, s, ArCD<sub>2</sub>CHO) ppm.

The product was not further characterised, but immediately reduced. Sodium borohydride (8mg., 0.22mmoles) was stirred in dry ethanol (3ml.) for 45 minutes. The crude deuterioaldehyde was dissolved in dry tetrahydrofuran (8ml.) in a flask fitted with septum and guard-tube, and the borohydride solution was added to this. The progress of the reaction was monitored by analytical thin layer chromatography, using chloroform-methanol (96:4) as eluant. After five minutes, hardly any substrate ( $R_f$  0.6) remained, while a spot corresponding to the unlabelled alcohol ( $R_f$  0.45) was visible. The reaction was quenched by adding 2N hydrochloric acid and extraction into ethyl acetate (3x 30ml.), which was dried (magnesium sulphate) and reduced *in vacuo* to yield a lime-green oil (183mg.).

This was purified by preparative thin layer chromatography using chloroform-methanol (96:4) as eluant. The most intense band under uv radiation was isolated to yield  $[1,3\text{-}^2\text{H}_4]\text{-7-benzyloxy-8-(2-hydroxyethyl)-5-methoxy-2-methyl-4-chromanone}$  (26) (70mg., 39%), as a clear oil which crystallised on standing. This was recrystallised from ethyl acetate to give white needles, m.p.128-135 C. (lit.<sup>3</sup>135-136 C).

-  $^1\text{H}$  N.M.R. (80 MHz.,  $\text{CDCl}_3$ ): 1.43 (3H, d,  $J=6.3\text{Hz.}$ , C- $\text{CH}_3$ ), 1.85 (1H, br s, -OH), 3.72 (2H, s,  $\text{ArCD}_2\text{CH}_2$ ), 3.81 (3H, s, - $\text{OCH}_3$ ), 4.45 (1H, q,  $J=6\text{Hz.}$ , 2-H), 5.12 (2H, s,  $\text{PhCH}_2$ -), 6.13 (1H, s, ArH), 7.36 (5H, s,  $\text{C}_6\text{H}_5$ ) ppm.

-  $^2\text{H}$  N.M.R. (31 MHz.,  $\text{CHCl}_3$ ): 2.57, 2.90 ppm.

- M.S. (m/z (%)) 346 ( $m^+$ , 13), 345 (14), 315 (18), 314 (20), 279 (15), 167 (37), 151 (30), 91 (100).



*[2'-<sup>2</sup>H<sub>1</sub>]-7-Benzoyloxy-8-(2-hydroxyethyl)-5-methoxy-2-methyl-4-chromanone (27)*

7-Benzoyloxy-8-(2-oxoethyl)-5-methoxy-2-methyl-4-chromanone (25) (35mg., 0.10mmoles) in dry tetrahydrofuran (1.5ml.) was reduced by sodium borodeuteride (Aldrich; 98% atom enriched) (10mg., 0.24mmoles) as described in the previous section. The product, a pale brown oil, was purified by preparative thin layer chromatography to give the desired product, *[2'-<sup>2</sup>H<sub>1</sub>]-7-benzoyloxy-8-(2-hydroxyethyl)-5-methoxy-2-methyl-4-chromanone (27)*, almost pure, as a white solid, (10mg., 29%).

- <sup>1</sup>H N.M.R. (80 MHz., CDCl<sub>3</sub>): 1.46 (3H, d, J=6.3Hz., C-CH<sub>3</sub>), 2.59 (2H, m, AB of ABX, J=7.6, 1.0Hz., 3-H<sub>2</sub>), 2.93 (d, J=7.5Hz., ArCH<sub>2</sub>CHD-), 2.94 (t, ArCH<sub>2</sub>CH<sub>2</sub>), 3.74 (m, ArCH<sub>2</sub>CHD-), 3.84 (3H, s, -OCH<sub>3</sub>), 4.50 (1H, dq, J=7, 1Hz., CHCH<sub>3</sub>), 5.15 (2H, s, PhCH<sub>2</sub>), 6.15 (1H, s, ArH), 7.38 (5H, s, C<sub>6</sub>H<sub>5</sub>) ppm.

- <sup>2</sup>H N.M.R. (55 MHz., CHCl<sub>3</sub>): 3.73 ppm.

- M.S. (m/z (%)) 343 (m<sup>+</sup>, 3), 311 (6), 287 (4), 256 (5), 255 (7), 221 (7), 94 (97), 91 (100).

*7-Benzoyloxy-5-methoxy-2-methyl-8-(2-(4-methylphenyl)-sulphonyloxyethyl)-4-chromanone*

7-Benzoyloxy-8-(2-hydroxyethyl)-5-methoxy-2-methyl-4-chromanone (24) (LL-D253α-7-*O*-benzyl ether) (118mg., 0.345mmoles) and p-toluenesulphonic acid (85mg., 0.49mmoles) were stirred together in refluxing benzene (25ml.) with the exclusion of moisture. After 15 hours the brown solution was allowed to cool, and reduced *in vacuo*. After preparative thin layer chromatography (chloroform-methanol (98:2)) the most intense band (R<sub>f</sub> 0.2), blue under uv

irradiation, was isolated, *7-benzyloxy-5-methoxy-2-methyl-8-(2-(4-methylphenyl)-sulphonyloxyethyl)-4-chromanone*. A small amount of starting material was also isolated. The product was recrystallised from ethyl acetate to give white needles, m.p. 83–85°C.

- Found: C, 65.5; H, 5.58%.  $C_{27}H_{28}O_7S$  requires C, 65.3; H, 5.68%.
- U.V. ( $\lambda_{\max}$ , (log $\epsilon$ )): 318 (3.62), 287 (4.22), 244 (4.04)nm.
- I.R. ( $\nu_{\max}^{\text{mull}}$ ): 1669 (s), 1586 (s), 1567 (s), 1302 (m), 1289 (m), 1262 (m), 1254 (m), 1205 (m), 1177 (s), 1172 (s), 1143 (m), 1111 (s).
- $^1\text{H}$  N.M.R. (80 MHz.,  $\text{CDCl}_3$ ): 1.33 (3H, d,  $J=6.2\text{Hz.}$ , C- $\text{CH}_3$ ) 2.34 (3H, s,  $\text{ArCH}_3$ ), 2.38–2.49 (2H, m, AB of ABX, 3-H), 2.94 (2H, t,  $J=6.8\text{Hz.}$ ,  $\text{ArCH}_2-$ ), 3.79 (3H, s,  $-\text{OCH}_3$ ), 4.10 (2H, t,  $J=6.8\text{Hz.}$ ,  $\text{ArCH}_2\text{CH}_2-$ ), 4.28–4.43 (1H, m, X of ABX,  $\text{CHCH}_3$ ), 5.05 (2H, s,  $\text{PhCH}_2$ ), 6.05 (1H, s,  $\text{ArH}$ ), 7.14 (2H, d,  $J=8.5\text{Hz.}$ , 2 of  $-\text{C}_6\text{H}_4\text{CH}_3$ ), 7.34 (5H, s,  $\text{C}_6\text{H}_5$ ), 7.58 (2H, d,  $J=8.4\text{Hz.}$ , 2 of  $\text{C}_6\text{H}_4\text{CH}_3$ ) ppm.
- M.S. ( $m/z$  (%)) 496 ( $m^+$ , 8), 234 (26), 192 (20), 107 (28), 92 (15), 91 (100).

***[1',2'- $^2\text{H}_1$ ]-7-Benzyloxy-5-methoxy-2-methyl-8-(2-(4-methylphenyl)-sulphonyloxyethyl)-4-chromanone (28)***

$[2'-^2\text{H}_1]$ -7-Benzyloxy-8-(2-hydroxyethyl)-5-methoxy-2-methyl-4-chromanone (27) (15mg., 0.04mmoles) and p-toluenesulphonic acid (9mg., 0.05mmoles) were dried thoroughly and stirred in refluxing benzene with the exclusion of moisture. Analytical thin layer chromatography (chloroform-methanol (98:2)) after 13 hours showed a major spot ( $R_f$  0.4) plus

starting material ( $R_f$  0.25). More *p*-toluenesulphonic acid (16mg., 0.08mmoles) was added. Analytical thin layer chromatography after a further 8 hours indicated that little starting material remained. The solution was reduced *in vacuo* to give a brown gum (68mg.) which was purified by preparative thin layer chromatography (Merck glass-backed silica plates; chloroform-methanol (96:4)). The main band ( $R_f$  0.8) was isolated (10mg., 46%), as a clear gum, *[1',2'-<sup>2</sup>H<sub>1</sub>]-7-benzyloxy-5-methoxy-2-methyl-8-(2-(4-methylphenyl)-sulphonyloxyethyl)-4-chromanone* (28).

- <sup>1</sup>H N.M.R. (80 MHz., CDCl<sub>3</sub>): 1.37 (3H, d,  $J=6.25\text{Hz.}$ , C-CH<sub>3</sub>), 2.98 (d,  $J=7.0\text{Hz.}$ , ArCH<sub>2</sub>CHD-), 2.98 (t,  $J=7.0\text{Hz.}$ , ArCH<sub>2</sub>CH<sub>2</sub>), 3.83 (3H, s, -OCH<sub>3</sub>), 4.13 (d,  $J=7.1\text{Hz.}$ , ArCHDCH<sub>2</sub>), 4.14 (t,  $J=7.0\text{Hz.}$ , ArCH<sub>2</sub>CH<sub>2</sub>), 4.25-4.50 (1H, m, X of ABX, CHCH<sub>3</sub>), 5.08 (2H, s, PhCH<sub>2</sub>-), 6.07 (1H, s, ArH), 7.18 (2H, d,  $J=8.6\text{Hz.}$ , 2 of C<sub>6</sub>H<sub>4</sub>CH<sub>3</sub>), 7.38 (5H, s, C<sub>6</sub>H<sub>5</sub>), 7.62 (2H, d,  $J=6.7\text{Hz.}$ , 2 of C<sub>6</sub>H<sub>4</sub>CH<sub>3</sub>) ppm.

- <sup>2</sup>H N.M.R. (55 MHz., CHCl<sub>3</sub>): 2.97, 4.13 ppm.

*[2'-<sup>2</sup>H<sub>1</sub>]-7-Benzyloxy-5-methoxy-2-methyl-8-(2-(4-methylphenyl)-sulphonyloxyethyl)-4-chromanone* (29)

*[2'-<sup>2</sup>H<sub>1</sub>]-7-Benzyloxy-8-(2-hydroxyethyl)-5-methoxy-2-methyl-4-chromanone* (27) (15mg., 0.044mmoles) and *p*-toluenesulphonyl chloride (27mg., 0.142mmoles) were stirred in pyridine (10 drops) at ambient temperature with the exclusion of moisture. After five hours the product was acidified (2N hydrochloric acid), giving a white precipitate, which was taken up in chloroform (2x20ml.). This was dried (magnesium sulphate) and <sup>1</sup>H N.M.R. indicated that the desired product was present, with minor impurities. After preparative thin layer chromatography (chloroform-methanol (97:3)), the

most intense band ( $R_f$  0.7) was isolated as a clear gum, *[2'-2 H<sub>1</sub>]-7-benzyloxy-5-methoxy-2-methyl-8-(2-(4-methylphenyl)-sulphonyloxyethyl)-4-chromanone* (29) (18mg., 83%).

-  $^1\text{H}$  N.M.R. (80 MHz.,  $\text{CDCl}_3$ ): 1.37 (3H, d,  $J=6.2\text{Hz.}$ , C- $\text{CH}_3$ ), 2.38 (3H, s,  $\text{ArCH}_3$ ), 2.45-2.55 (2H, m, AB of ABX, 3-H), 2.98 (d,  $J=7.0\text{Hz.}$ ,  $\text{ArCH}_2\text{CHD-}$ ), 2.98 (t,  $J=7.0\text{Hz.}$ ,  $\text{ArCH}_2\text{CH}_2\text{-}$ ), 3.82 (3H, s,  $-\text{OCH}_3$ ), 4.13 (brt,  $J=7.0\text{Hz.}$ ,  $\text{ArCH}_2\text{CHD-}$ ), 4.25-4.50 (1H, m, X of ABX, 2-H), 5.08 (2H, s,  $\text{PhCH}_2\text{-}$ ), 6.07 (1H, s,  $\text{ArH}$ ), 7.18 (2H, d,  $J=9.2\text{Hz.}$ , 2 of  $\text{C}_6\text{H}_4\text{CH}_3$ ), 7.37 (5H, s,  $\text{C}_6\text{H}_5$ ) 7.62 (2H, d,  $J=8.3\text{Hz.}$ , 2 of  $\text{C}_6\text{H}_4\text{CH}_3$ ) ppm.

-  $^2\text{H}$  N.M.R. (55 MHz.,  $\text{CHCl}_3$ ): 4.13 ppm.

*8-(2-Acetoxyethyl)-7-benzyloxy-5-methoxy-2-methyl-4-chromanone*

7-Benzyloxy-8-(2-hydroxyethyl)-5-methoxy-2-methyl-4-chromanone (24) (75mg., 0.22mmoles) was heated in glacial acetic acid at  $60^\circ\text{C}$  for 14 hours. The yellow solution was reduced *in vacuo* to give a brown gum. After preparative thin layer chromatography (chloroform-methanol (96:4)), the two main bands were isolated. That with the higher  $R_f$  (0.8) was the anticipated product. The material with the lower  $R_f$  (0.7) corresponded to an authentic sample of starting material. This was further heated in glacial acid for at  $60^\circ\text{C}$  for 48 hours. The product was worked up as above to yield, after preparative thin layer chromatography, starting material (23mg, ) and the anticipated product, *8-(2-acetoxyethyl)-7-benzyloxy-5-methoxy-2-methyl-4-chromanone* (21mg., 49mg. total, 58%, 84% corrected for recovered starting material). Recrystallisation from ether afforded clear blocky plates, m.p.  $99-101^\circ\text{C}$ .

- Found: C, 68.9; H, 6.35%.  $C_{22}H_{24}O_6$  requires C, 68.7; H, 6.29%.
- U.V. ( $\lambda_{\max}$ . (log $\epsilon$ )): 318 (3.72), 284 (4.35), 240 (4.10) nm.
- I.R. ( $\nu_{\max}^{\text{mull}}$ ): 1749 (s), 1672 (s), 1594 (s), 1238 (m), 1219 (s), 1141 (m), 1128 (s), 1078 (m), 814 (m)  $\text{cm}^{-1}$ .
- $^1\text{H}$  N.M.R. (80 MHz.,  $\text{CDCl}_3$ ): 1.44 (3H, d,  $J=6.3\text{Hz.}$ , C- $\text{CH}_3$ ) , 1.96 (3H, s, - $\text{COCH}_3$ ), 2.55 (2H, m, AB of ABX, 3-H), 2.95 (2H, t, 7.0Hz., Ar $\text{CH}_2$ ), 3.81 (3H, s, - $\text{CH}_3$ ), 4.16 (2H, t,  $J=7.0\text{Hz.}$ , Ar $\text{CH}_2\text{CH}_2$ -), 4.45 (1H, m, X of ABX, 2-H), 5.13 (2H, s,  $\text{PhCH}_2$ -), 6.11 (1H, s, ArH), 7.36 (5h, s,  $\text{C}_6\text{H}_5\text{CH}_2$ -) ppm.
- M.S. (m/z (%)) 384 ( $m^+$ , 3), 325 (10), 324 (40), 233 (27), 191 (14), 91 (100).

*[1',2'- $^2\text{H}_1$ ]-8-(2-Acetoxyethyl)-7-benzyloxy-5-methoxy-2-methyl-4-chromanone (30)*

$[2'-^2\text{H}_1]$ -7-Benzyloxy-5-methoxy-2-methyl-8-(2-(4-methylphenyl) sulphonyloxyethyl)-4-chromanone (29) (10mg., 0.020mmoles) was stirred in glacial acetic acid at ambient temperature with the exclusion of moisture for 15 hours. Difficulty was encountered in monitoring the reaction by analytical thin layer chromatography, as the presence of acetic acid caused streaking on the silica plate; however it was clear that some starting material remained. The reactants were stirred at  $60^\circ\text{C}$  for 20 hours.

The product was reduced *in vacuo* and taken up in chloroform (15ml.), This was washed with water (3x20ml.), dried (magnesium sulphate) and reduced *in vacuo* to yield a pale red gum. Preparative thin layer chromatography (chloroform) yielded starting material (3mg.) and the anticipated product,

*[1',2'-<sup>2</sup>H<sub>1</sub>]-8-(2-acetoxyethyl)-7-benzyloxy-5-methoxy-2-methyl-4-chromanone* (30) (4mg., 50%, 75% corrected), as a clear gum, with identical analytical thin layer chromatography properties to the unlabelled material.

- <sup>2</sup>H N.M.R. (55 MHz., CHCl<sub>3</sub>): 2.98, 4.18 ppm.

*[2'-<sup>2</sup>H<sub>1</sub>]-7-hydroxy-8-(2-hydroxyethyl)-5-methoxy-4-chromanone (31)*

A stirred, degassed solution of [2'-<sup>2</sup>H<sub>1</sub>]-7-benzyloxy-8-(2-hydroxyethyl)-5-methoxy-2-methyl-4-chromanone (27) (38mg., 0.11mmoles) in dry ethyl acetate containing a suspension of palladium-charcoal (10:90w/v, 20mg.), was hydrogenated at ambient temperature. The reaction was monitored by analytical thin layer chromatography (chloroform-methanol (96:4)). As starting material (R<sub>f</sub> 0.5) diminished in intensity, a spot corresponding to the anticipated product (R<sub>f</sub> 0.35) appeared.

After 3.5 hours the product was degassed and filtered through celite and reduced *in vacuo* to give a clear gum (36mg.) which was purified by preparative thin layer chromatography (chloroform-methanol (96:4)) to yield a white solid, *[2'-<sup>2</sup>H<sub>1</sub>]-7-hydroxy-8-(2-hydroxyethyl)-5-methoxy-2-methyl-4-chromanone* (31) (26mg., 93%).

- <sup>1</sup>H N.M.R. (60 MHz., (CD<sub>3</sub>)<sub>2</sub>CO): 1.4 (3H, d, J=6Hz., CH-CH<sub>3</sub>), 2.5 (2H, d, J=7Hz., 3-H), 2.8 (2H, d, J=6Hz., ArCH<sub>2</sub>CHD-), 3.6 (partially obscured, ArCH<sub>2</sub>CHD-), 3.75 (3H, s, -OCH<sub>3</sub>), 6.2 (1H, s, ArH) ppm.

- <sup>2</sup>H N.M.R. (55 MHz., (CH<sub>3</sub>)<sub>2</sub>CO, 2348 scans): 3.72 ppm.

### Attempted *in vivo* Scrambling of deuterium label in LL-D253 $\alpha$

Five 250ml. Erlenmeyer flasks, each containing 75ml. of medium were inoculated with a mycelial suspension of *Phoma pigmentivora* (QM502) as described previously, and incubated at 26°C in constant light on an orbital shaker. [2'-<sup>2</sup>H<sub>1</sub>]-7-Hydroxy-8-(2-hydroxyethyl)-5-methoxy-4-chromanone (31) (20mg.) was taken up in ethanol (0.5ml.) and sterile water (5ml.), and inoculated into one flask, after 60 hours' growth.

After a further 48 hours it was noted that the mycelia had darkened to a lesser extent than the control flasks.

Ten days after initial inoculation the flask's contents were worked up as described previously, to give a brown oil (207mg.), whose components were separated by preparative thin layer chromatography (chloroform-methanol (90:10), then acetone-chloroform (20:80)) to give a white solid (8mg.), partially deuteriated 7-hydroxy-8-(2-hydroxyethyl)-5-methoxy-2-methyl-4-chromanone (LL-D253 $\alpha$ ) (5).

- <sup>2</sup>H N.M.R. (55 MHz., (CH<sub>3</sub>)<sub>2</sub>CO, 76555 scans): 3.68 ppm.

*Crotonic anhydride*<sup>46</sup>

Dry Crotonic acid (40g., 0.47moles) was stirred under reflux in acetic anhydride (148g., 1.45moles) for 42 hours; precautions were taken to exclude moisture. Most acetic anhydride was removed by distillation. The residue was taken up in dry ether and shaken with anhydrous sodium carbonate. The solution was filtered and reduced *in vacuo*. The residue was distilled *in vacuo* using a Vigreux column; a clear liquid with a boiling range of 128–157°C (54mm.Hg) was collected, as almost pure (by n.m.r.) crotonic anhydride (20.1g., 56 %) (lit.<sup>45</sup>b.p. 128–130°C/ 19mm.Hg).

- <sup>1</sup>H N.M.R. (60 MHz., CDCl<sub>3</sub>): 1.38 (3H, dd, J=7, 1.5Hz., CHCH<sub>3</sub>), 5.87 (1H, dq, J=16, 1.5Hz., COCH), 7.11 (1H, dq, J=16, 7Hz., CHCH<sub>3</sub>) ppm.

*5,7-Dihydroxy-2-methyl-4-chromanone (37)***A: Using Crotonic anhydride/ Aluminium Trichloride**<sup>23</sup>

Anhydrous aluminium trichloride (2.18g, 17.3mmoles) and nitrobenzene (125 ml.) were added to phloroglucinol (30) (508mg., 4.03mmoles). After two hours' stirring crotonic anhydride (620mg., 4.03mmoles) was added to the green solution over thirty minutes. The solution was stirred at 40°C for 16 hours. The product was allowed to cool, then poured onto a mixture of ice and 4N hydrochloric acid (50ml. of each). The two layers were separated, and the organic layer further washed with hydrochloric acid. The nitrobenzene was removed by steam distillation.

An alternative isolation procedure, avoiding the need for a lengthy steam



distillation, was attempted. The nitrobenzene was extracted with 2N sodium hydroxide, which was back-extracted into ether. The dark brown alkaline solution was acidified (2N hydrochloric acid). The resulting yellow solution was extracted three times into ethyl acetate. This was combined, dried and reduced *in vacuo* to yield a brown solid. Yields for both isolation procedures were comparable; the latter required large quantities of solvent, and on some occasions it was very difficult to discern the interface between the organic and aqueous phases.

The product was purified by preparative thin layer chromatography (acetone-chloroform (20:80)), or column chromatography (acetone-ether (2:98)), to give a white solid. (0.88g., 26%). Recrystallisation from aqueous ethanol gave white needles, *5,7-dihydroxy-2-methyl-4-chromanone* (37), m.p. 176–178°C, (lit.<sup>23</sup> 176–177°C, lit.<sup>4</sup> 177–178°C).

- <sup>1</sup>H N.M.R. (60 MHz., (CD<sub>3</sub>)<sub>2</sub>CO) 0.95 (3H, d, J=6.5Hz., CHCH<sub>3</sub>), 2.03–2.25 (2H, AB of ABX, COCH<sub>2</sub>), 4.05 (1H, dq, J=6.5Hz., CHCH<sub>3</sub>), 5.38 (2H, s, ArH), 11.62 (1H, br s, exch., ArOH) ppm.

#### B: Crotonic acid/ Boron Trifluoride Etherate <sup>24</sup>

Dry phloroglucinol (2.662g., 21.13mmoles) and dry crotonic acid (1.909g., 22.20mmoles) were placed in a three-necked flask and dry nitrobenzene (100ml.) was added. This was stirred for one hour then cooled to 0°C. To the yellow suspension was added boron trifluoride etherate (12ml.) over twenty minutes. The resulting brown solution was heated at 110°C for 2.5 hours by which time a green solution had formed. This was poured into saturated sodium acetate solution (200ml.).

Sodium hydroxide solution (2N; 1000ml.) was added, and this was extracted into ether (four 500ml. portions). The alkaline solution was acidified (2N hydrochloric acid) and extracted three times into ethyl acetate. The combined organic extracts were dried and reduced *in vacuo* to give a brown gum. This was purified as described above to give a white solid (1.31g, 32%) with the same n.m.r. and thin layer chromatography characteristics as the material obtained in part A above.

**C: Crotonic Acid/ Methanesulphonic Acid/ Phosphorous Pentoxide** <sup>25</sup>

Methanesulphonic acid (80ml.) and phosphorous pentoxide (4.0g.) were heated to 70°C under a flow of dry nitrogen. Anhydrous phloroglucinol (6.18g., 49.0mmoles) and dry crotonic acid (4.225g., 49.1mmoles) were mixed and added. After 45 minutes' heating at 70°C this was allowed to cool, and the red solution was poured onto ice-water (600ml.), whereby an orange precipitate appeared. This was extracted into ether (three 250ml. portions) which was washed with water (two 150ml. portions) and brine (150ml.). The ethereal solution was dried and reduced *in vacuo* to yield a red oil (8.575g.).

This was purified by flash chromatography<sup>46</sup>, using as eluant 40-60 light petrol with an increasing proportion of ether. White crystals were obtained, of material with the same n.m.r. and thin layer chromatography characteristics as that described in A and B above, *5,7-dihydroxy-2-methyl-4-chromanone* (37) (5.76g., 60%).

- I.R. ( $\nu_{\text{max}}^{\text{mull}}$ ): 1630 (m), 1605 (s), 1304 (s), 1166 (s)  $\text{cm}^{-1}$ .
- $^1\text{H}$  N.M.R. (80 MHz.,  $\text{CDCl}_3$ ): 1.46 (3H, d,  $J=6.2\text{Hz.}$ ,  $\text{CHCH}_3$ ), 2.63-2.71 (2H, AB of ABX,  $\text{COCH}_2$ ), 4.53-4.64 (1H, dq,  $J=6.2\text{ Hz.}$ ,  $\text{CHCH}_3$ ), 5.92 (2H, s, ArH), 12.19 (1H, br s, exch., ArOH) ppm.

**D: "Thermal Reaction"**<sup>26</sup>

Dry Phloroglucinol (136mg., 1.08mmoles) and dry crotonic acid (95mg., 1.10mmoles) were slowly heated to 220°C under aflow of dry nitrogen gas for 3 hours. The product was allowed to cool. Analytical thin layer chromatography (acetone-chloroform (5:95)) indicated that only starting materials were present.

**E: Crotononitrile/benzyltrimethylammonium hydroxide**<sup>27</sup>

Phloroglucinol (500mg., 3.08mmoles) and crotononitrile were (492mg., 7.3mmoles) were stirred in the presence of benzyltrimethylammonium hydroxide ("Triton-B" catalyst) (40% aqueous solution) for 20 hours. The solution was acidified (dilute hydrochloric acid) and extracted into ether which was dried and reduced *in vacuo* to give an off-white solid, phloroglucinol (by n.m.r.). Increasing the reaction time or temperature had no effect on the result.

**6- And 8-acetyl-5,7-dihydroxy-2-methyl-4-chromanone (32)**

5,7-dihydroxy-2-methyl-4-chromanone (37) (100mg., 0.53mmoles) was stirred with acetic anhydride (5ml.) with the exclusion of moisture, and boron tribromide (5ml.) was cautiously added dropwise. This was stirred at 60°C; its progress monitored by analytical thin layer chromatography (acetone-chloroform(3:97)). After 6 hours the product was allowed to cool, then water (20ml.) was added, and this was extracted into ethyl acetate (two 50ml. portions). The latter was dried and reduced *in vacuo* to give a yellow solid (85mg.). Its components were separated by preparative thin layer chromatography (chloroform) to give two compounds with similar R<sub>f</sub> values, (0.7, 20mg.) and (0.65, 14mg.).

Higher R<sub>f</sub>

- M.p.: 151–155°C.
- Found: C, 59.9; H, 6.11%.  $C_{12}H_{12}O_5$  requires C, 61.0; H, 5.12%.
- $^1H$  N.M.R. (80 MHz.,  $CDCl_3$ ): 1.60 (3H, d,  $J=6.3Hz.$ ,  $CHCH_3$ ), 2.63 (3H, s,  $COCH_3$ ), 2.70 (2H, AB of ABX,  $COCH_2$ ) 4.70 (1H, m,  $CHCH_3$ ), 5.99 (1H, s, ArH), 12.66 (1H, s, exch., ArOH), 14.30 (1H, s, exch., ArOH) ppm.

Lower  $R_f$

- M.p. 117–120°C.
- Found: C, 61.0; H, 6.03; N, 0.34%.  $C_{12}H_{12}O_5$  requires C, 61.5; H, 5.12%.
- $^1H$  N.M.R. (80 MHz.,  $CDCl_3$ ): 1.51 (3H, d,  $J=6.3Hz.$ ,  $CHCH_3$ ), 2.70 (3H, s,  $COCH_3$ ), 2.69 (2H, AB of ABX,  $COCH_2$ ), 4.58 (1H, m, 1-CH), 5.94 (1H, s, ArH), 14.23 (1H, s, exch., ArOH), 14.48 (1H, s, exch., ArOH) ppm.

***7-Acetoxy-5-hydroxy-2-methyl-4-chromanone (41)***

5,7-dihydroxy-2-methyl-4-chromanone (37) (495mg., 2.55mmoles) was stirred with acetic anhydride (283mg., 2.77mmoles) and pyridine (5ml.) at ambient temperature for 6 hours. The product was poured on crushed ice, then extracted into chloroform (two 100ml. portions). This was washed with dilute hydrochloric acid (two 100ml. portions), dried and reduced *in vacuo* to give a brown solid (429mg.). This was purified by preparative thin layer

chromatography (acetone-chloroform (3:97)) to give white crystals ( $R_f$  0.7) of *7-acetoxy-5-hydroxy-2-methyl-4-chromanone* (41) (430mg., 71%). This was recrystallised from ether to give white needles, m.p. 110–112°C.

- Found: C, 60.9; H, 5.22%.  $C_{12}H_{12}O_4$  requires C, 61.0; H, 5.22%.
- U.V. ( $\lambda_{max}$ , (log $\epsilon$ )): 340 (3.24) 275 (3.83)nm.
- I.R. ( $\nu_{max}^{mull}$ ): 1754 (m), 1738 (m), 1653 (s), 1636 (s), 1583 (s), 1290 (m), 1218 (m), 1125 (s)  $cm^{-1}$ .
- $^1H$  N.M.R. (80 MHz.,  $CDCl_3$ ): 1.43 (3H, d,  $J=7.3Hz$ .,  $CHCH_3$ ), 2.20 (3H, s,  $COCH_3$ ), 2.57–2.68 (2H, AB of ABX,  $COCH_2$ ), 4.50 (1H, dq,  $J=7.3$ , 1.9Hz.,  $CHCH_3$ ), 6.15 (1H, d,  $J=2.1Hz$ ., ArH), 6.18 (1H, d,  $J=2.1Hz$ ., ArH), 11.78 (1H, s, exch., ArOH) ppm.
- M.S. ( $m/z$ (%)) 236 ( $m^+$ , 44), 194 (91), 179 (21), 153 (24), 152 (100).

#### Attempted Fries Rearrangement of *7-Acetoxy-5-hydroxy-2-methyl-4-chromanone*

Boron trifluoride etherate (2ml.) was added dropwise to *7-acetoxy-5-hydroxy-2-methyl-4-chromanone* (41) (53mg., 0.22mmoles). This was stirred at 60°C for 40 minutes with the exclusion of moisture. The product was poured on ice, then extracted into methylene chloride (two 100ml. portions). The resulting solution was dried and reduced *in vacuo* to give a yellow solid which was readily soluble in chloroform. analytical thin layer chromatography (acetone-chloroform (5:95)) indicated a complex mixture of products, and that neither starting material nor the anticipated product were present. This was confirmed by the  $^1H$  n.m.r. spectrum of the crude mixture,

which also suggested that 5,7-dihydroxy-2-methyl-4-chromanone was present.

**Attempted Photo-Fries Rearrangement of 7-acetoxy-5-hydroxy-2-methyl-4-chromanone<sup>28</sup>**

The title compound (41) (46mg., 0.19mmoles) was dissolved in ethyl acetate (100ml.) in a quartz immersion well, and dry nitrogen was bubbled through the solution. This was irradiated, with water cooling, using standard photochemical apparatus (125W.) for nine hours. Removal of solvent *in vacuo* gave pale brown crystals, of starting material, 7-acetoxy-5-hydroxy-2-methyl-4-chromanone, by analytical thin layer chromatography (acetone-chloroform (3:97);  $R_f = 0.6$ ).

**Attempted Photo-Fries Rearrangement of 7-acetoxy-5-hydroxy-2-methyl-4-chromanone in the presence of Potassium Carbonate**

The title compound (48mg., 0.20mmoles) was irradiated in ethyl acetate as described above; in addition potassium carbonate (120mg.) was present, and the resulting suspension was stirred magnetically. After ten hours this was filtered and reduced *in vacuo* to give a clear gum (130mg.), mainly starting material by thin layer chromatography and n.m.r.

**2,4,6-Trihydroxyacetophenone (38)<sup>31</sup>**

Phloroglucinol dihydrate was dried in a vacuum oven for fourteen hours at 120°C to remove water of crystallisation. Fresh zinc chloride was dried in a vacuum dessicator. Acetonitrile was dried over phosphorus pentoxide as described in reference . All glassware was dried overnight at 120°C prior to use.

Phloroglucinol (30.1g., 240mmoles), zinc chloride (6.05g., 45mmoles) and acetonitrile (25ml., 19.6g., 480mmoles) in sodium-dried ether(400ml.) were

cooled to 0°C, with stirring, in a three-necked flask. Precautions were taken to exclude moisture. Dry hydrogen chloride gas was bubbled vigorously through the solution. Excess hydrogen chloride was vented from the reaction flask through a beaker of water. The colour of the solution turned yellow within thirty minutes. A pale yellow precipitate formed on the inside of the gas delivery tube and frequently had to be removed. Once the solution was saturated with hydrogen chloride, usually within two hours, the flask was sealed and refrigerated overnight. More pale yellow precipitate formed. Dry hydrogen chloride was again bubbled through the solution until saturated, and then sealed and refrigerated for three days.

The ether was decanted off the precipitate, which was further washed with fresh ether. It was then dissolved in water (1000ml.) and refluxed for two hours. The yellow solution was allowed to cool for five minutes, and decolourising charcoal (2g.) was added. The solution was heated until boiling, then filtered immediately. A precipitate formed from the filtrate, which was allowed to cool overnight. The resulting thick pale yellow slurry was filtered and recrystallised from water to give white needles of *2,4,6-trihydroxyacetophenone* (38) (34.0g., 85%), m.p. 215–220°C (lit.<sup>31</sup> 218–219°C).

– <sup>1</sup>H N.M.R.: (60 MHz., (CD<sub>3</sub>)<sub>2</sub>CO) 1.92 (3H, s, COCH<sub>3</sub>), 5.30 (2H, s, ArH), 8.70 (1H, br s, exch., ArH) 10.97 (2H, br s, exch., ArH).

#### *2-Hydroxy-4,6-dimethoxyacetophenone (42)*

A: Using Iodomethane

All glassware was oven-dried prior to use. Dry

2,4,6-trihydroxyacetophenone (38) (4.295g., 25.6mmoles), anhydrous potassium carbonate (17.65g., 128 mmoles) and dry acetone (200ml.) were stirred mechanically in a three-necked flask, with the exclusion of moisture. The acetone was heated under reflux, and iodomethane (8.25ml., 18.8g., 125mmoles) was added dropwise over thirty minutes. The reaction was monitored by preparative thin layer chromatography (chloroform). After hours, most of the starting material ( $R_f$  0.1; purple under ultraviolet (354nm.) irradiation) had gone, and two lower polarity spots ( $R_f$  0.7 and 0.8; blue and purple respectively under ultraviolet irradiation) were evident. The flask's contents were allowed to cool, and were filtered. the filtrate was washed with acetone and the combined filtrate (400ml.) was reduced *in vacuo*. The residue was partitioned between dilute hydrochloric acid and ethyl acetate. The latter was dried and reduced *in vacuo* to yield a yellow solid.

Purification by flash chromatography was attempted, using a 10mm. diameter column and elution with 30/40 petrol containing an increasing proportion of ether. Impurities were removed, but separation of the two main components was incomplete. However in some cases partial concentration of the eluant resulted in formation of yellow blocky prisms, and later of white needles, which were separated manually. Attempted fractional crystallisation from ether yielded yellow needles; filtration and slow removal of solvent yielded white needles, *2-hydroxy-4,6-dimethoxyacetophenone* (42) (3.89g., 77%), m.p. 80–81°C (lit.<sup>32</sup> 85–88°C).

–  $^1\text{H}$  N.M.R. (60 MHz.,  $\text{CDCl}_3$ ): 2.60 (3H, s,  $\text{ArCH}_3$ ) ppm, 3.83 (3H, s,  $\text{ArOCH}_3$ ), 3.87 (3H, s,  $\text{ArOCH}_3$ ), 5.98 (1H, d,  $J=2\text{Hz.}$ ,  $\text{ArH}$ ), 6.11 (1H, d,  $J=2\text{Hz.}$ ,  $\text{ArH}$ ), 14.15 (1H, s, *exch.*,  $\text{ArOH}$ ) ppm.



The yellow crystals were recrystallised from ether to yield *2-hydroxy-4,6-dimethyl-3-methylacetophenone* (47) (g.), m.p. 145–146°C (lit.<sup>32</sup> 141–142°C).

– <sup>1</sup>H N.M.R. (200 MHz., CDCl<sub>3</sub>): 1.98 (3H, s, ArCH<sub>3</sub>), 2.58 (3H, s, COCH<sub>3</sub>), 3.86 (6H, s, ArOCH<sub>3</sub>), 5.91 (1H, s, ArH), 14.05 (1H, s, exch, ArOH) ppm.

#### B: Using dimethyl sulphate <sup>33</sup>

Dry 2,4,6-trihydroxyacetophenone (10.0g., 59.5mmoles) and anhydrous potassium carbonate (65g., 470mmoles) in dry acetone (500ml.) were stirred mechanically under reflux with the exclusion of moisture. Dimethyl sulphate (12.4ml., 16.5g., 131mmoles) was added dropwise; heating and stirring were continued for twelve hours. The solution had changed from yellow to pale white in colour. The suspension was reduced *in vacuo* to a white solid, and partitioned between ether and water. The organic layer was extracted into 2N sodium hydroxide, whereby a yellow colour formed. This was cautiously poured onto 6N hydrochloric acid, producing a white precipitate, *2-hydroxy-4,6-dimethoxyacetophenone* (42) (8g., 69%). The ether layer was found to contain *2,4,6-trimethoxyacetophenone*, m.p. 100–103°C (lit.<sup>33</sup> 104°C).

– <sup>1</sup>H N.M.R. (60 MHz., CDCl<sub>3</sub>): 2.52 (3H, s, ArCH<sub>3</sub>), 3.79 (6H, s, OCH<sub>3</sub>), 3.83 (3H, s, OCH<sub>3</sub>), 6.02 (2H, s, ArH) ppm.

#### *Crotonyl Chloride* <sup>47</sup>

Crotonic acid (40.5g., 0.39 moles) was dried, then stirred under reflux with sodium-dried light petrol. Thionyl chloride (50ml., 81.5g., 0.69moles) was added

over 10 minutes, then the reactants were refluxed for four hours. Precautions were taken to exclude moisture. The product was allowed to cool, then volatile components were removed *in vacuo*. The product was purified by distillation through a Vigreux column; material with a boiling range of 119–123°C (lit.<sup>47</sup> 124–126°C) was retained, *crotonyl chloride* (36.3g., 67.4%). This was re-refrigerated until used.

- <sup>1</sup>H N.M.R. (60 MHz., CDCl<sub>3</sub>): 1.94 (3H, dd, J=7Hz., J=1.8Hz, CHCH<sub>3</sub>), 5.97 (1H, dq, J=15Hz., J=1.8Hz., COCH), 7.15 (1H, dq, J=15Hz., J=7Hz., CHCH<sub>3</sub>).

#### *2-crotonoxy-4,6-dimethoxyacetophenone (43)*

2-Hydroxy-4,6-dimethoxyacetophenone (167mg., 0.85mmoles) was refluxed for 45 hours with crotonyl chloride (1ml., 1.09g., 10.4mmoles) and a (1 inch) strip of magnesium ribbon in dry toluene (50ml.). The solution was allowed to cool, and, after removal of the magnesium, reduced *in vacuo*. The resulting black gum was partitioned between dilute potassium carbonate solution and ether (two 50ml. portions). The latter were dried and reduced *in vacuo* to give a brown gum (166mg.). This was purified by preparative thin layer chromatography (ether) to give a white solid, *2-crotonoxy-4,6-dimethoxyacetophenone* (43) (3.08g., 55%). Recrystallisation from ethanol gave clear blocky prisms (m.p. 100–102°C).

- Found: C, 63.6; H, 6.14%. C<sub>14</sub>H<sub>16</sub>O<sub>5</sub> requires C, 63.6; H, 6.10%.
- U.V. (λ<sub>max</sub>. (logε)): 286 (sh., 3.60), 260 (3.88), 238 (3.91) nm.
- I.R. (ν<sub>max</sub><sup>mull</sup>): 1730 (s), 1682 (s), 1611 (s), 1343 (s), 1303 (m), 1252 (s), 1226 (s), 1201 (m), 1154 (s), 1096 (s), 1079 (s), 971

(s), 894 (m), 845 (m), 721 (m)  $\text{cm}^{-1}$ .

-  $^1\text{H}$  N.M.R. (200 MHz.,  $\text{CDCl}_3$ ): 1.92 (3H, dd,  $J=6.9, J=1.7\text{Hz}$ .,  $-\text{CHCH}_3$ ), 2.43 (3H, s,  $\text{ArCH}_3$ ), 3.77 (3H, s,  $\text{ArOCH}_3$ ), 3.81 (3H, s,  $\text{ArOCH}_3$ ), 5.97 (1H, dq,  $\text{COCHCHCH}_3$ ), 6.23 (1H, d,  $J=2.3\text{Hz}$ , ArH), 6.34 (1H, d,  $J=2.3\text{Hz}$ ., ArH), 7.12 (1H, dq,  $J=15.5, 6.93\text{Hz}$ .,  $-\text{CHCH}_3$ ) ppm.

- M.S. ( $m/z(\%)$ ) 262 ( $\text{M}^+$ , 50), 247 (100), 219 (33), 205 (19), 121 (12), 69 (12).

#### Attempted Reduction of 2-Crotonoxy-4,6-dimethoxyacetophenone

Sodium borohydride (325mg., 8.68mmoles) was stirred in dry ethanol for one hour. 2-crotonoxy-4,6-dimethoxyacetophenone (500mg., 1.89mmoles) was dissolved in dry tetrahydrofuran (10ml.) and sodium borohydride solution (4ml.) added. The reaction was monitored by analytical thin layer chromatography (chloroform). After stirring at ambient temperature for fifteen minutes, a product started to appear ( $R_f$  0.3), of lower polarity than the starting material ( $R_f$  0.5). After nineteen hours, the solution was acidified and the resulting precipitate was taken up in ethyl acetate (25ml.). This was washed with water (25ml.), dried and reduced *in vacuo* to give a brown gum, which partly solidified on standing. Its components were separated by preparative thin layer chromatography to give mainly 1-(2-Hydroxy-4,6-dimethoxyphenyl)-ethanol (48) and 2-Ethyl-3,5-dimethoxyphenol (49) (by analytical thin layer chromatography and n.m.r.).

1-(2-Hydroxy-4,6-dimethoxyphenyl)-ethanol (48)

-  $^1\text{H}$  N.M.R. (220 MHz.,  $\text{CDCl}_3$ ): 1.45 (3H, d,  $J=6.5\text{Hz.}$ ,  $\text{CH}(\text{OH})\text{CH}_3$ ), 3.10 (1H, brs, exch.,  $\text{CH}(\text{OH})\text{CH}_3$ ), 3.72 (3H, s,  $\text{ArOCH}_3$ ), 3.73 (3H, s,  $\text{ArOCH}_3$ ), 5.41 (1H, q,  $J=6.5\text{Hz.}$ ,  $\text{CH}(\text{OH})\text{CH}_3$ ), 5.97 (1H, d,  $J=2.3\text{Hz.}$ , ArH), 6.04 (1H, d,  $J=2.3\text{Hz.}$ , ArH), 8.80 (1H, brs, exch., ArOH) ppm.

2-Ethyl-3,5-dimethoxyphenol (49)

-  $^1\text{H}$  N.M.R. (80 MHz.,  $\text{CDCl}_3$ ): 1.08 (3H, t,  $J=7.5\text{Hz.}$ ,  $\text{CH}_2\text{CH}_3$ ), 2.58 (2H, q,  $J=7.6\text{Hz.}$ ,  $\text{CH}_2\text{CH}_3$ ), 3.71 (3H, s,  $\text{OCH}_3$ ), 3.76 (3H, s,  $\text{OCH}_3$ ), 4.54 (1H, br s, exch., ArOH), 6.03 (1H, d,  $J=2.5\text{Hz.}$ , ArH), 6.08 (1H, d,  $J=2\text{Hz.}$ , ArH) ppm.

The title compound (505mg., 2.02mmoles) in dry ethanol (20ml.) was added to a suspension of sodium borohydride adsorbed on silica (10%, 762mg., 2mmoles) in dry tetrahydrofuran (10ml.). This was stirred at ambient temperature under argon. Progress was monitored by analytical thin layer chromatography; after 43 hours mainly starting material (rf 0.45) was present, plus a small amount of of product (rf 0.6). The suspension was stirred under reflux for 3 hours, causing the proportion of this product to increase. After cooling , the suspenion was filtered, and the filtrate reduced *in vacuo* to give a yellow oil; a portion of this was purified by preparative thin layer chromatography to give a mixture of starting material (43) and 2-hydroxy-4,6-dimethoxyacetophenone (42), by analytical thin layer chromatography and n.m.r.

The title compound (199mg., 0.796mmoles) was stirred with sodium cyanoborohydride (48mg., 0.77mmoles) in dry methanol (5ml.). This was stirred

at ambient temperature for 17 hours. The product was reduced *in vacuo* and partitioned between brine and ether. The ethereal solution was washed with sodium hydrogen carbonate solution (5%), dried and reduced *in vacuo* to give a white solid. Its components were separated by preparative thin layer chromatography to give, by analytical thin layer chromatography and n.m.r., the same two compounds as mentioned above.

#### **Attempted reduction of 2-hydroxy-4,6-dimethoxyacetophenone**

The title compound (505mg., 2.58mmoles) was reacted with sodium borohydride (155 mg., 4.10mmoles) in dry ethanol (6ml.) as described for its crotonate ester above. The main products were a mixture of the hydroxyethyl- and ethyl phenols described above.

The title compound (505mg., 2.58mmoles) was also reacted with sodium borohydride adsorbed on silica (8%; 980mg, 2.5mmoles borohydride) in dry ethanol as described above. Again starting material plus the same mixture of reduction products were obtained.

#### **Attempted hydrogenation of 2-hydroxy-4,6-dimethoxyacetophenone**

The title compound (2.24g., 11.4mmoles) was stirred for 18 hours with palladium on charcoal (10%; 155mg.) in ethanol. The solution was filtered and reduced *in vacuo* to give a white solid, starting material (by analytical thin layer chromatography and n.m.r.).

The same procedure was repeated using glacial acetic acid as solvent, with the same result. The solution was repeatedly reduced *in vacuo* and carbon tetrachloride added to remove acetic acid as its azeotrope. The resulting white solid appeared to be starting material by analytical thin layer chromatography and n.m.r..

Attempted Sodium/Ethanol Reduction of 2-Hydroxy-  
4,6-dimethoxyacetophenone

The title compound (500mg., 2.55mmoles) was dissolved in dry ethanol and sodium (57.5mg., 2.5mmoles) was added. The solution was stirred under reflux for three hours, then reduced *in vacuo*. The residue was partitioned between dilute hydrochloric acid and ethyl acetate. The latter was dried and reduced *in vacuo* to give a white solid, starting material by analytical thin layer chromatography and n.m.r..

*2-Ethyl-3,5-dimethoxyphenol (49)*<sup>38</sup>

Zinc (4.45g.) and mercuric chloride (220mg.) were shaken with dilute hydrochloric acid (25ml.) for 5 minutes. The aqueous solution was then decanted off, and replaced with a mixture of water and concentrated hydrochloric acid (150ml. of each). 2-hydroxy-4,6-dimethoxyacetophenone (5g., 25.5mmoles) was added, and the suspension stirred under reflux for four hours. Initially not all the substrate dissolved, but as it was used up during the course of the reaction, a brown oil formed above the aqueous layer.

After cooling, the supernatant liquids were decanted off any remaining zinc/mercury residues. The former were saturated with sodium chloride and extracted three times into ether (200ml. total) which was dried and reduced *in vacuo* to give a yellow oil, almost pure (by n.m.r.) *2-ethyl-3,5-dimethoxyphenol (49)*. This was purified by distillation *in vacuo* to give a clear oil, b.p. 111-113°C, 2mm.Hg (lit. 164°C, 20mm.Hg).

- I.R. ( $\nu_{\text{max}}^{\text{neat}}$ ): 3420 (s), 1509 (m), 1248 (m), 1218 (m), 1202 (m),  
1151 (s), 1119 (s), 1053 (m), 978 (m), 808 (m)  $\text{cm}^{-1}$ .

- $^1\text{H}$  N.M.R. (60 MHz.,  $\text{CDCl}_3$ ): 1.05 (3H, t,  $J=7\text{Hz.}$ ,  $\text{CH}_2\text{CH}_3$ ), 2.52 (2H, q,  $J=7\text{Hz.}$ ,  $\text{CH}_2\text{CH}_3$ ), 3.65 (3H, s,  $\text{OCH}_3$ ), 3.72 (3H, s,  $\text{OCH}_3$ ), 5.36 (1H, br s, exch.,  $\text{ArOH}$ ), 5.97 (1H, d,  $J=2\text{Hz.}$ ,  $\text{ArH}$ ), 6.07 (1H, d,  $J=2\text{Hz.}$ ,  $\text{ArH}$ ).

*(4-Ethyl-3,5-dimethoxyphenyl) crotonate (55)*

2-Ethyl-3,5-dimethoxyphenol (49) (2.0g., 11.0mmoles) and crotonyl chloride (2.0ml., mg., mmoles) were stirred under reflux with a (1 inch) strip of magnesium ribbon in dry toluene (50ml.), with the exclusion of moisture. After 48 hours, the magnesium was removed, and the organic solution was washed with water, then (2%) sodium hydroxide solution. After drying this was reduced *in vacuo* to give a brown oil, pure (by n.m.r.) *(4-ethyl-3,5-dimethoxyphenyl) crotonate* (55) (2.19mg., 80%). This was further purified by analytical thin layer chromatography (ethyl acetate-petrol (30:70)), to give a clear oil which slowly darkened on standing.

- Found: C, 65.6; H, 7.20.  $\text{C}_{14}\text{H}_{18}\text{O}_4$  requires C, 67.2; H, 7.25%.
- U.V. ( $\lambda_{\text{max}}$ , ( $\log \epsilon$ )): 278 (3.39), 239 (3.61) nm.
- I.R. ( $\nu_{\text{max}}^{\text{neat}}$ ): 2975 (m), 2947 (m), 2883 (m), 2846 (m), 1740 (s), 1656 (m), 1611 (s), 1590 (s), 1496 (m), 1455 (m), 1440 (m), 1425 (m), 1308 (m), 1292 (m), 1240 (s), 1219 (s), 1200 (s), 1151 (s), 1109 (s), 1050 (s), 993 (m), 968 (m), 830 (m),  $\text{cm}^{-1}$ .
- $^1\text{H}$  N.M.R. (60 MHz.,  $\text{CDCl}_3$ ): 0.93 (3H, t,  $J=7.5\text{Hz.}$ ,  $\text{CH}_2\text{CH}_3$ ), 1.87 (3H, dd,  $J=7, 1.5\text{Hz.}$ ,  $\text{CHCH}_3$ ), 2.51 (2H, q,  $J=7.5\text{Hz.}$ ,  $\text{CH}_2\text{CH}_3$ ), 3.68 (3H, s,  $\text{OCH}_3$ ), 3.72 (3H, s,  $\text{OCH}_3$ ), 6.02 (1H, dq,  $J=16, 1.5\text{Hz.}$ ,  $\text{COCH}$ ), 6.24 (1H, d,  $J=2\text{Hz.}$ ,  $\text{ArH}$ ), 6.34 (1H, d,  $J=2\text{Hz.}$ ,

ArH), 7.18 (1H, dq, J=16, 7Hz., CHCH<sub>3</sub>).

*1-(3-Ethyl-2-hydroxy-4,6-dimethoxyphenyl) but-2-en-1-one (56)*

**A: Fries rearrangement of (4-Ethyl-3,5-dimethoxyphenyl) crotonate** <sup>41</sup>

The title compound (263mg., 1.05mmoles) and anhydrous aluminium trichloride (84mg., 1.38mmoles) were stirred at 150°C for two hours with the exclusion of moisture. The resulting red gum was allowed to cool, and ice was added. The product was extracted into ethyl acetate, which was dried and reduced *in vacuo* to give a red oil. This contained (by analytical thin layer chromatography (chloroform-methanol (98:2)) starting material and a product with the same properties as that obtained from the "Magnesium-directed Friedel-Crafts reaction" described in the following paragraph, *1-(3-Ethyl-2-hydroxy-4,6-dimethoxyphenyl) but-2-en-1-one (56)* (97mg., 37%).

**B: "Magnesium-directed Friedel-Crafts reaction"** <sup>42</sup>

Magnesium was dessicator-dried overnight, Bromoethane was dried over magnesium sulphate, filtered and distilled from phosphorus pentoxide in the absence of moisture. All glassware was dried overnight at 120°C. Precautions were taken to exclude moisture.

Magnesium (133mg., 5.47mmoles) was stirred in dry ether (15ml.) and bromoethane (0.38ml., 0.55g., 5.1mmoles) was added over five minutes. 2-ethyl-3,5-dimethoxyphenol (913mg., 5.93mmoles) in dry ether (15ml.) was added over ten minutes, then the ether was reduced *in vacuo* at ambient temperature to give a pale yellow solid. Toluene (30ml.), freshly distilled from sodium was added. To the yellow slurry was added crotonyl chloride (0.485ml., 0.53g., 5.06mmoles) in dry toluene (7ml.) over ten minutes, and the



mixture was stirred overnight.

Analytical thin layer chromatography after twenty hours (acetone–light petroleum (25:75) showed one major spot, purple under ultraviolet irradiation ( $R_f$  0.6) and others ( $R_f$  0.55 and 0.5) corresponding to starting materials.

The product was poured onto ammonium chloride solution, then extracted into ether (three 50ml. portions). The yellow solution was reduced *in vacuo* to give a brown oil. Purification by preparative thin layer chromatography (acetone: light petroleum (25:75)) yielded a yellow oil as the major component ( $R_f$  0.75), along with some of its cyclisation product (see below). The former was crystallised from ether to give yellow needles, *1-(3-Ethyl-2-hydroxy-4,6-dimethoxyphenyl) but-2-en-1-one* (56) (609g., 48%), m.p. 98–105°C.

– Found: C, 67.7; H, 7.29%.  $C_{12}H_{14}O_4$  requires C, 67.2; H, 7.25%.

– U.V. ( $\lambda_{max}$ , (log $\epsilon$ )): 315 (4.28), 242 (4.12), 224 (sh., 3.90) nm.

– I.R. ( $\nu_{max}^{mull}$ ): 1745 (m), 1615 (s), 1590 (s), 1147 (s), 1138 (s), 966 (m)  $cm^{-1}$ .

–  $^1H$  N.M.R. (80 MHz.,  $CDCl_3$ ): 1.06 (3H, t,  $J=7.39Hz$ ,  $ArCH_2CH_3$ ), 1.94 (3H, dd,  $J=3.83, 1.51Hz$ ,  $CHCH_3$ ), 2.59 (2H, q,  $J=7.39Hz$ ,  $CH_2CH_3$ ), 3.86 (3H, s,  $OCH_3$ ), 3.87 (3H, s,  $OCH_3$ ), 5.93 (1H, s,  $ArH$ ), 7.04– 7.32 (2H, m,  $COCH + CHCH_3$ ), 13.94 (1H, exch., s,  $ArOH$ ) ppm.

– M.S. ( $m/z$ (%)): 250 ( $M^+$ , 32), 235 (24), 182 (48), 181 (20), 167 (56), 69 (100).

*5,7-Dimethoxy-8-ethyl-2-methyl-4-chromanone (57)*

1-(3-Ethyl-2-hydroxy-4,6-dimethoxyphenyl) but-2-en-1-one (56) (58mg., 0.26mmoles) was dissolved in deuteriochloroform, and shaken with a dilute solution of sodium deuteriomethoxide in deuterium oxide. Any changes were monitored by n.m.r.; little change had occurred within two hours and the solution was stirred overnight at ambient temperature. Its n.m.r. spectrum indicated the appearance of signals at 1.5 and 4.4 ppm., accompanied by diminution of signals at 1.9 and 7.0–7.3 ppm.

After 94 hours the product was further extracted into chloroform. This was dried and reduced *in vacuo* to give a light-brown solid, which was purified by preparative thin layer chromatography (acetone-petrol (25:75)) to give *5,7-dimethoxy-8-ethyl-2-methyl-4-chromanone* (57) as a pale yellow solid (46mg., 80%), m.p. 128–130°C. This was recrystallised from ether to give white needles, m.p. 130–131.

– Found: C, 67.3; H, 7.30%.  $C_{14}H_{18}O_4$  requires C, 67.2; H, 7.25%.

– U.V. ( $\lambda_{\max}$ . (log $\epsilon$ )): 318 (3.64), 284 (4.22), 239 (4.04) nm.

– I.R. ( $\nu_{\max}^{\text{mull}}$ ): 1679 (s), 1598 (s), 1573 (s), 1342 (s), 1312 (m), 1269 (s), 1211 (m), 1148 (m), 1311 (s), 1082 (m), 799 (m), 720 (m)  $\text{cm}^{-1}$ .

–  $^1\text{H}$  N.M.R. (80 MHz.,  $\text{CDCl}_3$ ): 1.03 (3H, t,  $J=7.36\text{Hz.}$ ,  $\text{CH}_2\text{CH}_3$ ), 1.44 (3H, d,  $J=6.26\text{Hz.}$ ,  $\text{CHCH}_3$ ), 2.55 ( $\text{CH}_2\text{CH}_3$ ), 2.55 (2H, q,  $-\text{COCH}_2-$ ), 3.86 (3H, s,  $\text{ArOCH}_3$ ), 3.89 (3H, s,  $\text{ArOCH}_3$ ), 4.44 (1H, dq,  $J=7.0, 1.6\text{Hz.}$ ,  $\text{CHCH}_3$ ), 6.06 (1H, s, ArH) ppm.

– M.S. ( $m/z(\%)$ ): 250 ( $\text{M}^+$ , 96), 235 (52), 221 (33), 193 (100), 151

(134), 150 (22).

*2-Ethyl-3,5-dihydroxyphenol (C-ethyl phloroglucinol) (61)*

*2-hydroxy-4,6-dihydroxyacetophenone* (38) (4.50g., 26.8mmoles) was reduced with activated zinc in hydrochloric acid as described for its dimethyl ether above, to give a brown oil (2.55g., 61%). This was refluxed in benzene for 16 hours, using a Dean-Stark trap to remove any water as its azeotrope, to give a pale-brown solid, almost pure by n.m.r. and analytical thin layer chromatography, *2-ethyl-3,5-dihydroxyphenol (C-ethyl phloroglucinol) (61)* (2.55g., 52%), m.p. 134–137°C. (lit. 165°C from anisole).

–  $^1\text{H}$  N.M.R. (60 MHz.,  $\text{CDCl}_3$ ): 0.55 (3H, t,  $J=7\text{Hz.}$ ,  $\text{CH}_2\text{CH}_3$ ), 2.05 (2H, q,  $J=7\text{Hz.}$ ,  $\text{CH}_2\text{CH}_3$ ), 5.47 (2H, s, ArH), 7.40 (3H, s, exch., ArOH) ppm.

*5,7-dihydroxy-8-ethyl-2-methyl-4-chromanone (60)*

Phosphorous pentoxide (250mg.) was heated to 90°C in methanesulphonic acid (5ml.) under a flow of nitrogen. To this was added a mixture of *2-ethyl-3,5-dihydroxyphenol (C-ethyl phloroglucinol) (61)* (308mg., 2.0mmoles) and crotonic acid (168mg., 2.0mmoles), which was stirred at 90°C for one hour. After cooling, ice water was added, and the product was extracted into ether (two 50ml. portions). The ethereal solution was washed with water, then brine, dried and reduced *in vacuo* to give a red oil. Its components were separated by preparative thin layer chromatography (ethyl acetate–light petrol (30:70)), to give four compounds ( $R_f$  0.6–0.8) which were yellow when visualised with

concentrated sulphuric acid (10%) in ethanol. Starting material ( $R_f$  0.5) was also present.

Compound A was purple under u.v. irradiation and gave yellow prisms from ether, m.p. 126–129°C.

- Found: C, 65.6; H, 6.17%.  $C_{16}H_{18}O_5$  requires C, 66.2; H, 6.25%.
- U.V. ( $\lambda_{max}$  (log $\epsilon$ )): 349 (3.82), 286 (4.53), 238 (4.17)nm.
- I.R. ( $\nu_{max}^{null}$ ): 1790 (s), 1644 (s), 1631 (s), 1603 (m), 1342 (s), 1297 (m), 1250 (m), 1231 (m), 1170 (m), 1137 (s), 1098 (s)  $cm^{-1}$ .
- $^1H$  N.M.R. (220 MHz.,  $CDCl_3$ ): 1.10 (3H, t,  $J=7.4Hz.$ ,  $CH_2CH_3$ ), 1.17 (3H, dd,  $J=7.1, 2.1Hz.$ ,  $ArCHCH_3$ ), 1.51 (3H, d,  $J=6.3Hz.$ ,  $CHCH_3$ ), 2.63 (2H, q,  $J=7.4Hz.$ ,  $CH_2CH_3$ ), 2.66–2.72 (4H, m,  $COCH_2$ ), 3.38–3.47 (1H, m,  $ArCHCH_3$ ), 4.54 (1H, dq,  $J=6.3, 2.0Hz.$ ,  $CHCH_3$ ), 12.08 (1H, d,  $J=5.4Hz.$ , exch.,  $ArOH$ ) ppm.
- M.S. (m/z (%)): 290 (74), 276 (18), 275 (100), 234 (11), 233 (93), 191 (11).

Compound B appeared orange under u.v. irradiation and gave white needles from ether, m.p. 149–151°C.

- Found: C, 66.4; H, 6.33%.  $C_{16}H_{18}O_5$  requires C, 66.2; H, 6.25%.
- I.R. ( $\nu_{max}^{null}$ ): 1770 (m), 1631 (s), 1244 (s), 1155 (s), 1130 (s),

1097 (m)  $\text{cm}^{-1}$ .

- $^1\text{H}$  N.M.R. (200 MHz.,  $\text{CDCl}_3$ ): 1.07 (3H, dt,  $J=7.4$ ,  $0.7\text{Hz.}$ ,  $\text{CH}_2\text{CH}_3$ ), 1.19 (3H, d,  $J=7.1\text{Hz.}$ ,  $\text{ArCHCH}_3$ ), 1.51 (3H, dd,  $J=6.3$ ,  $0.5\text{Hz.}$ ,  $\text{CHCH}_3$ ), 2.60 (2H, q,  $J=7.4\text{Hz.}$ ,  $\text{CH}_2\text{CH}_3$ ), 2.60–2.72 (4H, m, 2x  $\text{COCH}_2$ ), 3.45–3.50 (1H, m,  $\text{ArCHCH}_3$ ), 4.52 (1H, dq,  $J=9.1$ ,  $6.1\text{Hz.}$ ,  $\text{CHCH}_3$ ), 12.13 (1H, s, exch.,  $\text{ArOH}$ ) ppm.
- M.S. ( $m/z$  (%)): 290 (55), 275 (100), 247 (30), 233 (55), 205 (21), 191 (17).

Compound C appeared purple under u.v. irradiation and gave small white blocks from ether, m.p.  $181\text{--}183^\circ\text{C}$ .

- Found: C, 64.8; H, 6.45%.  $\text{C}_{12}\text{H}_{14}\text{O}_5$  requires C, 64.8; H, 6.35.
- I.R. ( $\nu_{\text{max}}^{\text{mull}}$ ): 1631 (s), 1580 (s), 1310 (m), 1122 (m)  $\text{cm}^{-1}$ .
- $^1\text{H}$  N.M.R. (80 MHz.,  $(\text{CD}_3)_2\text{CO}$ ) 1.05 (3H, t,  $J=7.4\text{Hz.}$ ,  $\text{CH}_2\text{CH}_3$ ), 1.49 (3H, d,  $J=6.3\text{Hz.}$ ,  $\text{CHCH}_3$ ), 2.56 (2H, q,  $J=7.2\text{Hz.}$ ,  $\text{CH}_2\text{CH}_3$ ): 2.55–2.70 (2H, AB of ABX,  $\text{COCH}_2$ ), 3.10 (brs, exch.,  $\text{ArOH}$ ), 4.57 (1H, dq,  $J=8.6$ ,  $6.4\text{Hz.}$ ,  $\text{CHCH}_3$ ), 5.99 (1H, s,  $\text{ArH}$ ), 12.08 (1H, s, exch.,  $\text{ArOH}$ ) ppm.
- M.S. ( $m/z$  (%)): 222 ( $\text{M}^+$ , 45), 208 (12), 207 (100), 165 (45), 123 (15).

#### Compound D

- $^1\text{H}$  N.M.R. (80 MHz.,  $(\text{CD}_3)_2\text{CO}$ ): 1.06 (3H, t,  $J=7.4\text{Hz.}$ ,  $\text{CH}_2\text{CH}_3$ ),

1.43 (3H, d,  $J=6.3\text{Hz}$ .,  $\text{CHCH}_3$ ), 2.56 (2H, q,  $J=7.2\text{Hz}$ .,  $\text{CH}_2\text{CH}_3$ ),  
2.58–2.70 (2H, AB of ABX,  $\text{COCH}_2$ ), 3.04 (1H, brs, exch.,  $\text{ArOH}$ ),  
4.54 (1H, dq,  $J=9.2, 6.3\text{Hz}$ .,  $\text{CHCH}_3$ ), 5.99 (1H, s,  $\text{ArH}$ ), 12.42 (1H,  
s, exch.,  $\text{ArH}$ ) ppm.

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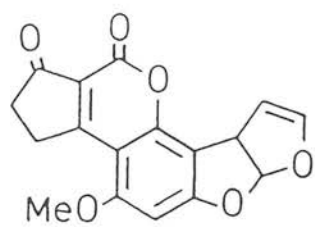


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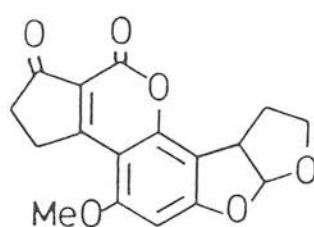
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## CHAPTER 3

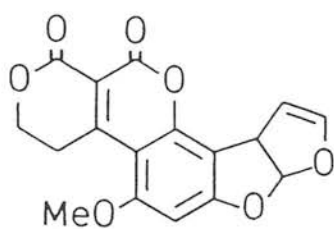
THE BIOSYNTHESIS OF AFLATOXIN B<sub>1</sub>



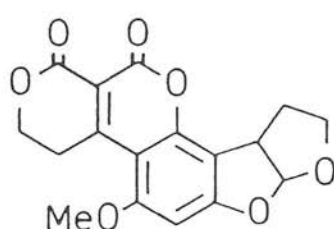
aflatoxin B<sub>1</sub> (1)



aflatoxin B<sub>2</sub> (2)



aflatoxin G<sub>1</sub> (3)



aflatoxin G<sub>2</sub> (4)

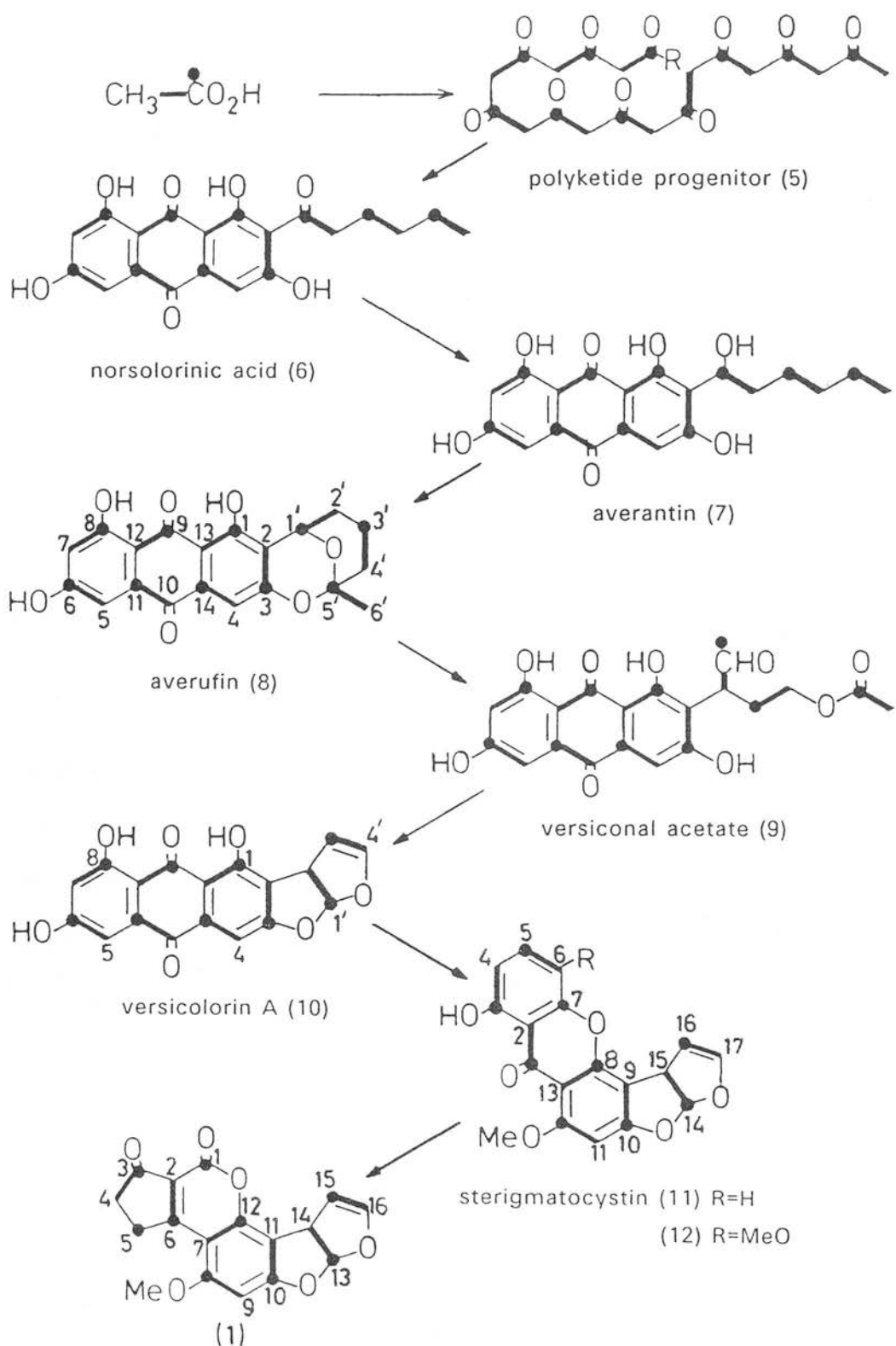
### 3.1. Introduction

#### 3.1.1. Background Information

The mycotoxins are a range of fungal-derived secondary metabolites, which cause diseases in both animals and humans<sup>1</sup>. Evidence exists for the effects of mycotoxins since biblical times<sup>2</sup>. The effects of ergotism which caused many thousands of deaths in Europe have been well documented from the middle ages<sup>3</sup>. For many other diseases caused by mycotoxins, only in this century has the individual fungus been identified as the aetiological agent. From 1960 onwards there has been an upsurge not only in the awareness of the medical and economic threat posed by mycotoxins, but also in the development, across a range of disciplines, of quantitative scientific techniques used in the identification of mycotoxins and determining their biochemical action. Many reviews covering all aspects of mycotoxin research are available<sup>1,4,5,6</sup>.

It is no coincidence that the beginning of this period of intensive research coincides with the discovery of aflatoxin B<sub>1</sub>, perhaps the most studied of all mycotoxins.<sup>6,7</sup> The aflatoxins (1)–(4) are produced by *Aspergillus flavus* and *Aspergillus parasiticus*; the former was implicated in "Turkey X" disease<sup>8</sup>, which in 1960 caused the death of 100,000 turkey poults in Great Britain<sup>9</sup>. The presence of histopathological lesions in these<sup>10</sup>, and other<sup>11</sup>, animals was discovered. The common factor was found to be Brazilian groundnut meal<sup>12</sup> which was shown to be contaminated by *A. flavus*<sup>13</sup>. It was subsequently shown to contaminate a range of crops during growth, harvesting, processing, storage and shipment<sup>5</sup>. Aflatoxin poisoning has been suggested as the cause of previously reported diseases of then unknown aetiology.

Elegant studies by Buchi and coworkers led in 1965 to the structural elucidation of aflatoxin B<sub>1</sub><sup>14</sup>, which was shown to be a methoxycoumarin with



Scheme 1: The proposed aflatoxin biosynthetic pathway

fused bisfuranoid and cyclopentenone moieties (1). Its toxic, carcinogenic, mutagenic and teratogenic properties have all been demonstrated; aflatoxin B<sub>1</sub> is the most potent known hepatocarcinogen for several animal species. It has also been implicated in human liver cancer<sup>15</sup>. Its highly carcinogenic nature is attributed to the bisfuranoid double bond, known to be activated *in vivo* to the corresponding epoxide which binds to guanine residues in DNA<sup>16</sup>.

Early biosynthetic studies suggested a polyketide origin for aflatoxin B<sub>1</sub><sup>17</sup>. Buchi and his coworkers confirmed this by incorporation of <sup>14</sup>C-labelled acetates into (1) followed by extensive degradative efforts<sup>18</sup>. Twelve of the sixteen nuclear carbon atoms were unambiguously pinpointed, each with consistent levels of radioactivity, and are in accord with the labelling pattern shown for aflatoxin B<sub>1</sub> (1) in scheme 1.

After 1970, the use of *A. parasiticus* mutants (ultra-violet radiation or chemically induced) or the metabolic inhibitor "dichlorvos" led to the accumulation of a range of potential biosynthetic precursors to the aflatoxins, as shown in scheme 1. Norsolorinic acid (6), the first stable intermediate in the pathway, isolated from *A. versicolor* in 1967<sup>19</sup> and latterly from an *A. parasiticus* mutant in 1971<sup>20</sup>, incorporated <sup>14</sup>C label from acetate. Radioactivity from norsolorinic acid labelled in this way was incorporated into aflatoxin B<sub>1</sub> by *A. parasiticus* wild-type<sup>21</sup>. The next purported intermediate, averantin (7), the dihydro-analogue of norsolorinic acid, was first isolated from *A. versicolor* in 1966<sup>22</sup>, and later from an *A. parasiticus* mutant. Averantin incorporated radioactivity from acetate, and the resulting labelled averantin was incorporated into aflatoxin B<sub>1</sub> by *A. parasiticus* wild-type<sup>23</sup>. The internal ketal averufin (8), first isolated from *A. versicolor* in 1963<sup>24</sup>, and later from a high-yielding *A. parasiticus* mutant<sup>25</sup>, was shown to incorporate radioactivity from acetate<sup>26,27,28</sup> and from averantin<sup>23</sup>. Averufin itself <sup>14</sup>C-labelled from

acetate was incorporated into aflatoxin B<sub>1</sub> by *A. parasiticus* wild-type<sup>26,28,29</sup>.

Treatment of *A. parasiticus* wild-type with the insecticide "dichlorvos" (dimethyl-2,2-dichlorovinyl phosphate) lowered levels of aflatoxin production and led to the accumulation of versiconal acetate (9), the first metabolite on the pathway with a branched side-chain<sup>30,31,32,33</sup>. It incorporates radioactivity from acetate<sup>27</sup>, and this label is incorporated from versiconal acetate into aflatoxin B<sub>1</sub> by wild-type<sup>33</sup> and averufin-producing cultures of *A. parasiticus*, and by *A. flavus*<sup>27</sup>. Versicolorin A (10), the first metabolite on the pathway containing the bisfuranoid moiety, was first isolated from *A. versicolor* in 1966<sup>34</sup>, and was latterly isolated from a further *A. parasiticus* mutant<sup>35</sup>. It incorporates radioactivity from acetate<sup>27,36</sup> and again versicolorin A radiolabelled from acetate was incorporated into aflatoxin B<sub>1</sub> by the *A. parasiticus* wild-type<sup>36</sup>, and by the averufin-accumulating mutant<sup>37</sup>. In contrast to the above compounds, the last proposed intermediate on the pathway is the xanthone sterigmatocystin (11)<sup>38,39,40,41</sup>, produced from a wide range of *Aspergillus*, and other, species<sup>42</sup> and was first isolated in 1954. *A. versicolor* incorporates radioactivity from acetate<sup>27</sup>, averufin, versiconal acetate and versicolorin A<sup>43</sup> into sterigmatocystin. Sterigmatocystin labelled in this way is converted into aflatoxin B<sub>1</sub> by the *A. parasiticus* wild-type<sup>44,45</sup> and by the averufin-producing mutant<sup>27</sup>.

From the above, and other results, the biosynthetic sequence shown in scheme 1 was constructed. This order was reinforced by kinetic pulse-labelling<sup>46</sup>. This technique is useful for the detection of transient intermediates, and the determination of their relative order in a biosynthetic sequence<sup>47</sup>. The organism under scrutiny is fed with a radiolabelled precursor (in this case, acetate), and at various stages its metabolism is rapidly quenched and assayed by chromatographic separation coupled with autoradiography. The



results from this study are in agreement with the proposed biosynthetic scheme, but may raise doubt as to the true intermediacy of sterigmatocystin, whose appearance was first detected *after* that of aflatoxin B<sub>1</sub>.

A major drawback of the above results is that the incorporation of "biosynthetically labelled" materials is prone to misinterpretation, due to the possibility of prior degradation to acetate<sup>48</sup>. Rigorous proof of intermediacy is only supplied by *specific* incorporation of isotopic label.

It is clear from scheme 1 that many interesting mechanistic problems are raised. One of the most intriguing is the transformation of the linear side-chain of averufin (8) to the branched side-chain of versiconal acetate (9), apparently involving a "1,2-aryl shift". In addition this transformation involves insertion of oxygen between the 4' and 5' atoms of the side-chain, suggesting the possibility of a Baeyer-Villiger oxidation from (formally) a ketone (8) to an ester (9). Conversion of versicolorin A (10) to sterigmatocystin (11) entails loss of the C-6 phenolic hydroxy group, the timing or purpose of which is uncertain, and loss of the C-10 (carbonyl) carbon of versicolorin A. The biosynthesis of xanthenes by oxidative cleavage of an anthraquinone precursor followed by decarboxylation has been well documented for *other* systems<sup>49</sup>, but not until recently for sterigmatocystin. The conversion of sterigmatocystin (11) or a closely-related metabolite (e.g. its 6-methoxy analogue (12)<sup>50,51</sup>) to aflatoxin B<sub>1</sub> must entail ring cleavage and rearrangement with decarboxylation to the cyclopentene-coumarin structure<sup>52</sup>.

In the late 1970s efforts by Steyn and his coworkers<sup>6</sup> led to unambiguous <sup>13</sup>C-NMR assignments of averufin (8)<sup>53</sup>, versiconal acetate (9)<sup>54</sup>, versicolorin A (10)<sup>55</sup>, sterigmatocystin (11) and aflatoxin B<sub>1</sub> (1)<sup>56</sup>, and related metabolites. This led to the incorporation of [1-<sup>13</sup>C]-<sup>57</sup> and [2-<sup>13</sup>C]-acetates into averufin (8)<sup>53</sup>,

versiconal acetate (9)<sup>30,31,54</sup>, versicolorin A (10)<sup>55</sup>, sterigmatocystin (11)<sup>58</sup> and aflatoxin B<sub>1</sub> (1)<sup>59,60</sup>, the results of which confirmed the common polyketide origin for these metabolites. Incorporation of [1,2-<sup>13</sup>C<sub>2</sub>]- (*doubly*) labelled acetate into (8)<sup>28</sup>, (9)<sup>53</sup>, (10)<sup>30,54</sup>, (11) and (1)<sup>60</sup> combined with analysis of the interacetate coupling pattern has revealed a common polyketide folding pattern as shown for (5) in scheme 1.

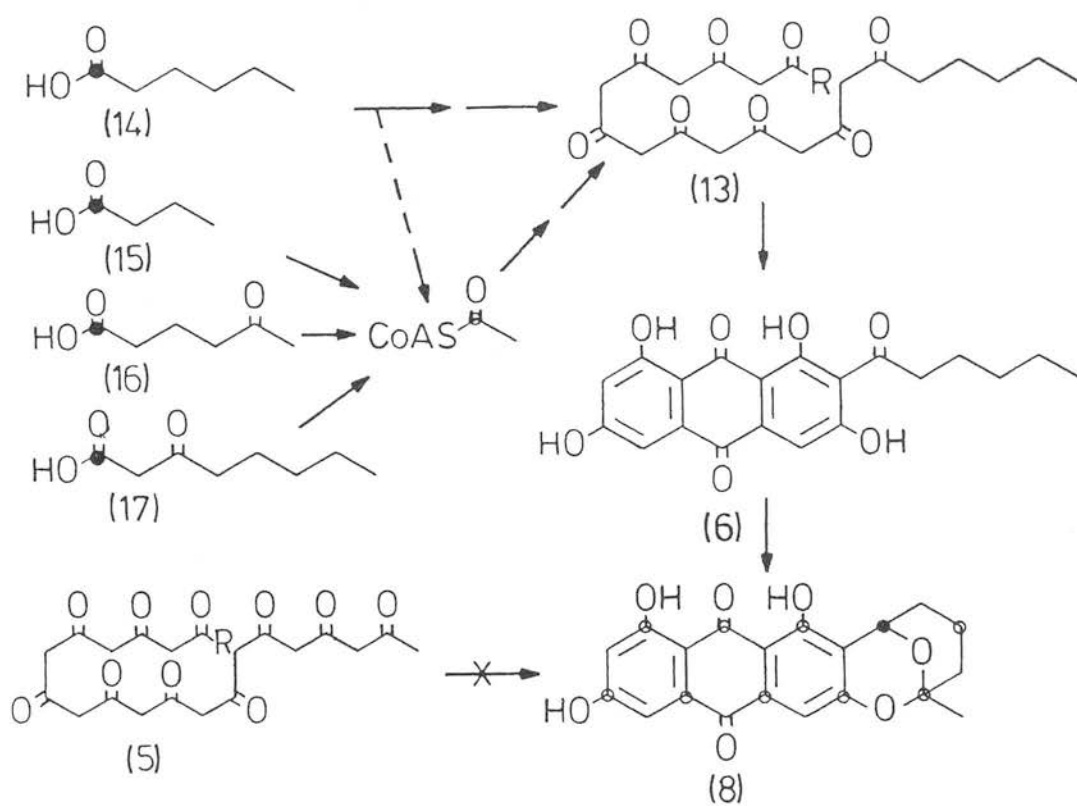
A comprehensive review of aflatoxin biosynthesis by Steyn covers relevant work until 1980. Subsequent studies have seen the use of variously and multiply labelled acetates and advanced precursors<sup>61</sup>, in order to probe some of the problems mentioned above. These studies, and their mechanistic implications, will now be discussed in more detail.

### 3.1.2. Hexanoate Incorporation into Averufin

So far no mention of the early stages of biosynthesis, leading to norsolorinic acid (6), has been made. This reflects the notion held until recently that the enzyme-bound polyketide is fully oxygenated<sup>62</sup> (as for example in (5)) before secondary modification takes place. However recent studies indicate that processes such as reduction, dehydration and hydrogenation, analogous to fatty acid biosynthesis, occur while the polyketide chain is being constructed<sup>63</sup>.

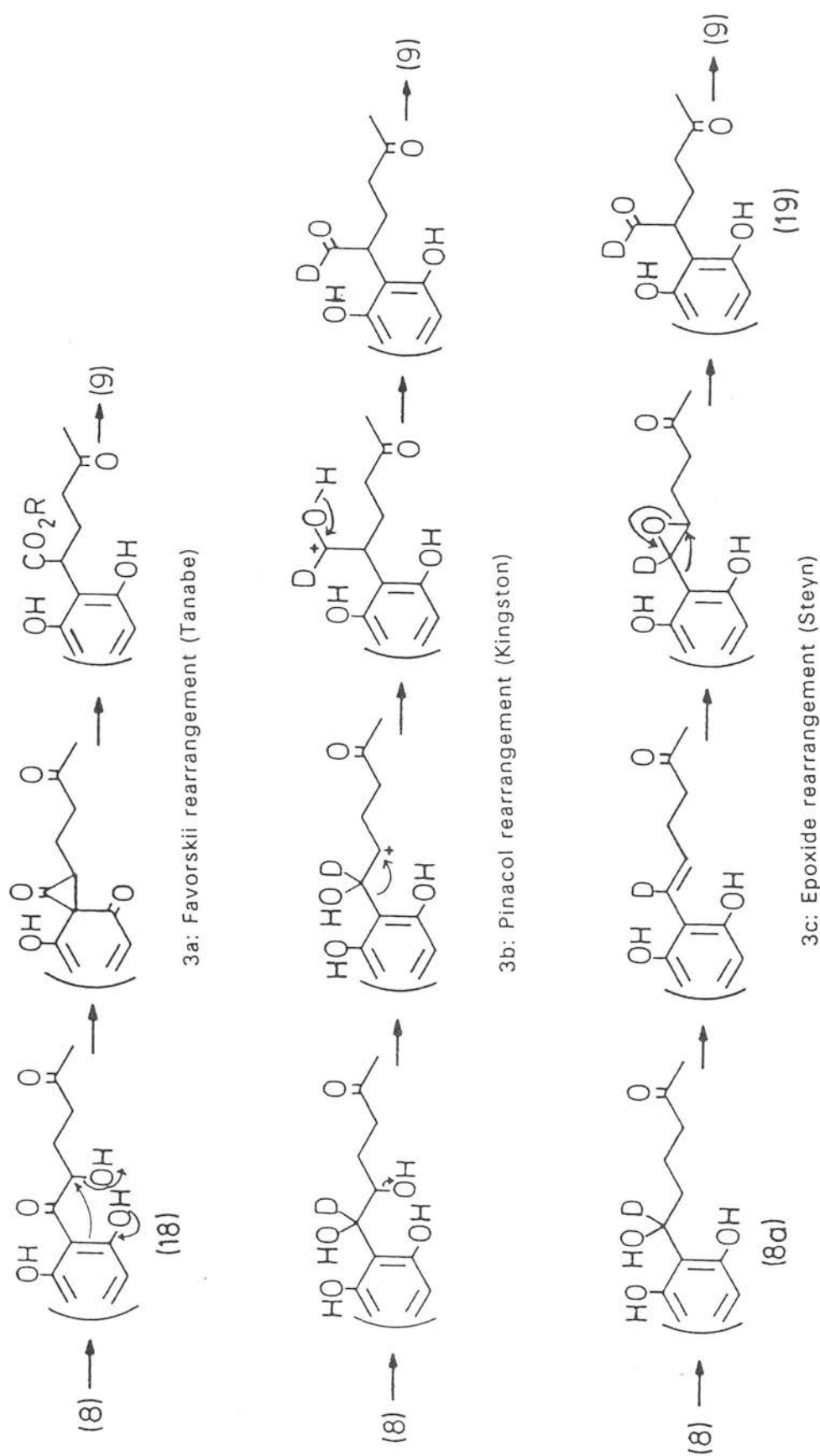
It is noteworthy that the first isolable intermediate on the aflatoxin pathway, norsolorinic acid (6), has a highly reduced side-chain, and that the pattern of deuterium labelling of the averufin side chain from  $[^2\text{H}_3]$ - and  $[^2\text{H}_3, 1\text{-}^{13}\text{C}]$ -acetate is analogous to that of fatty acid biosynthesis<sup>64,65</sup>. In addition, Townsend<sup>61,66</sup>, and Sankawa<sup>64</sup>, have noted that the C-5' (methylene) carbon of norsolorinic acid, which is derived from the carbonyl carbon of acetate, is subsequently oxidised to the (formally) carbonyl carbon of averufin. In studies relating to a limited number of polyketides which retain oxygen attached to the C-1 carbon derived from the starter acetate unit, *that* oxygen is acetate derived<sup>67</sup>. Further, it was noted by Holker that incorporation of radioactivity into the four bisfuranoid carbons of sterigmatocystin (11) was equal but significantly lower than incorporation into the xanthone nucleus<sup>17</sup>. With these anomalies in mind, Townsend suggested that hexanoate, rather than acetate, forms the starter unit for the aflatoxin pathway. The possibility that the polyketide pathway may employ starter units other than acetate was first proposed by Birch<sup>62</sup>. However there is little experimental evidence to support this.

In 1983 Townsend reported the *specific* incorporation of  $[1\text{-}^{13}\text{C}]$ -hexanoic acid (14) into averufin by *A. parasiticus*, accompanied by a smaller level of secondary incorporation of acetate labelled by catabolism of hexanoate<sup>66</sup>. In

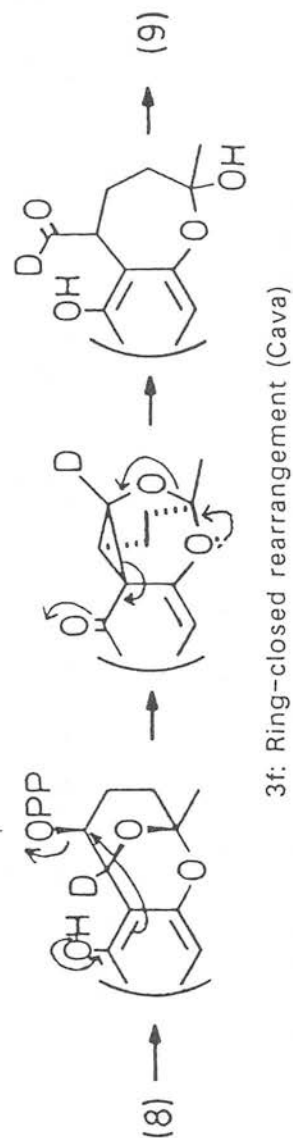
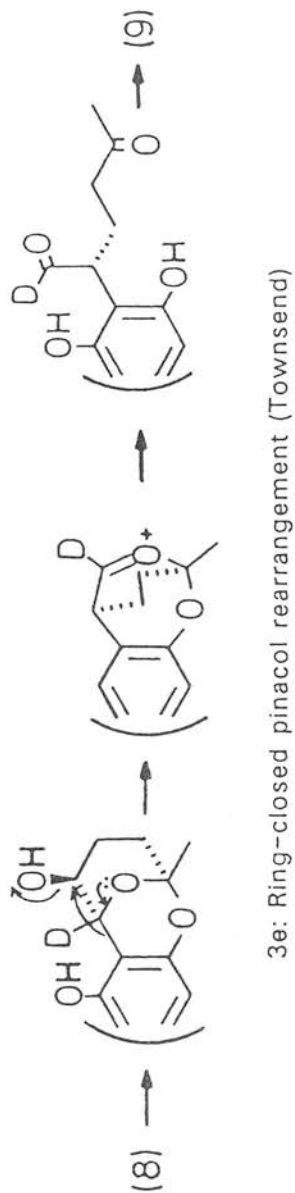
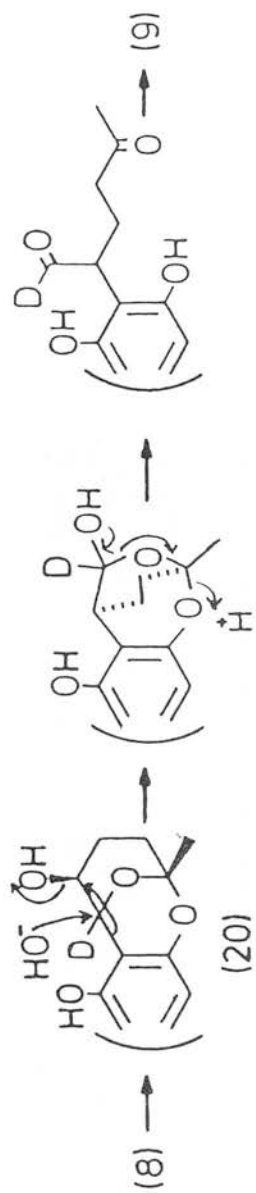


Scheme 2: The role of hexanoate in averufin biosynthesis

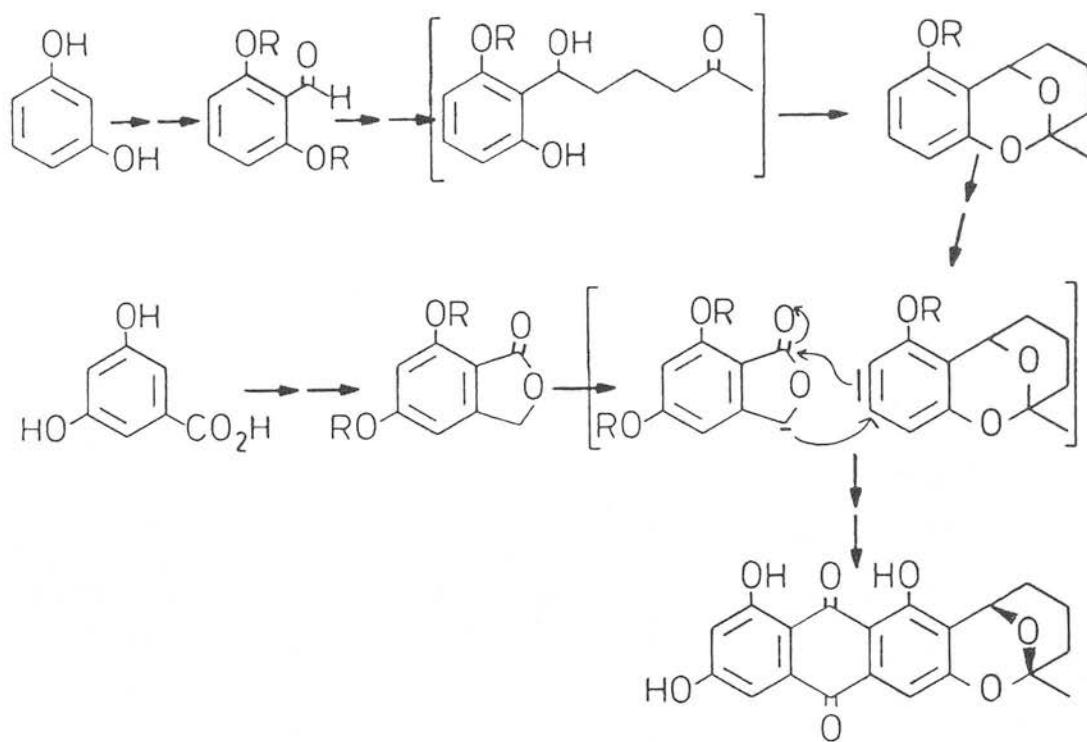
parallel studies however, [1-<sup>13</sup>C]-butyric acid (15), [1-<sup>13</sup>C]-5-oxohexanoic acid (16), and [1-<sup>13</sup>C]-3-oxooctanoic acid (17) were also incorporated into averufin but *not* specifically, only through secondary incorporation via acetate (scheme 2)<sup>68</sup>. This significant result suggests that hexanoate, formed elsewhere by fatty acid synthetase, form the starter unit for an *octaketide*. However it is also possible that a polyketide synthase produces a reduced 6-carbon segment, which may exchange with free hexanoyl CoA before continuing synthesis of a *decaetide* (scheme 2).



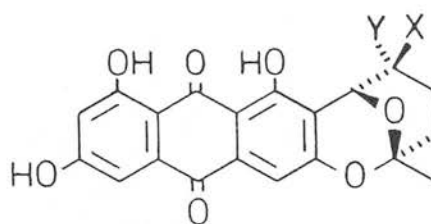
Scheme 3: Postulated mechanisms for the 1,2-aryl shift during versiconal acetate formation



Scheme 3 (continued)



Scheme 4: Townsend's averufin synthesis<sup>72</sup>;  $R = \text{CH}_3\text{OCH}_2-$



(20)  $X = \text{OH}, Y = \text{H}$

(21)  $X = \text{H}, Y = \text{OH}$

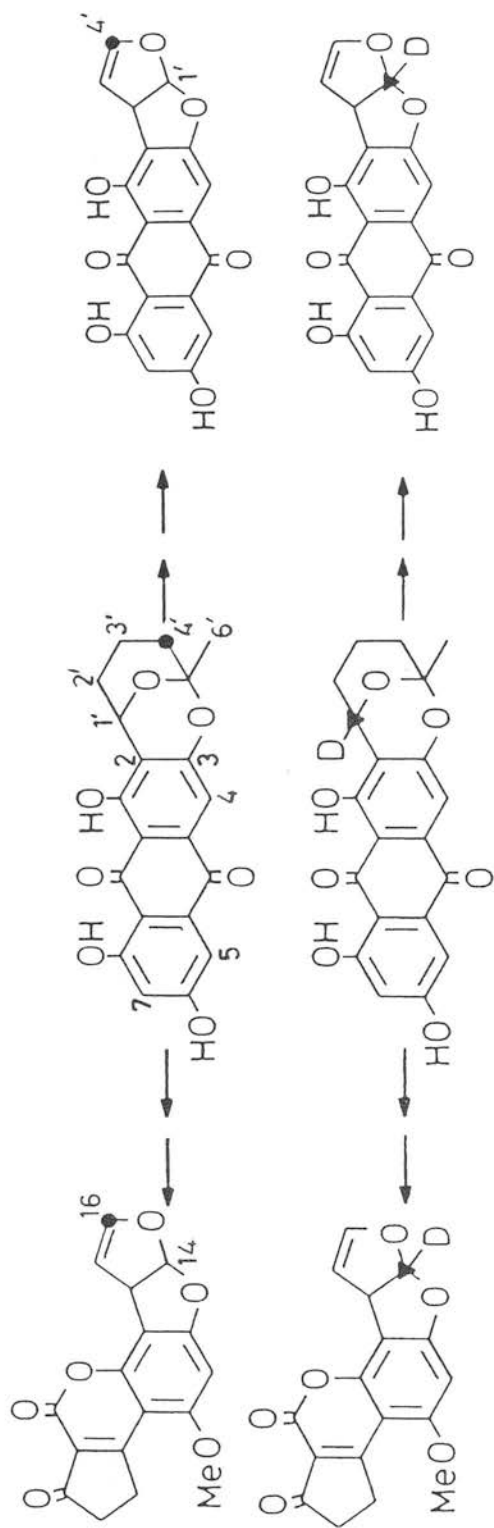


### 3.1.3. Rearrangement of the averufin side-chain

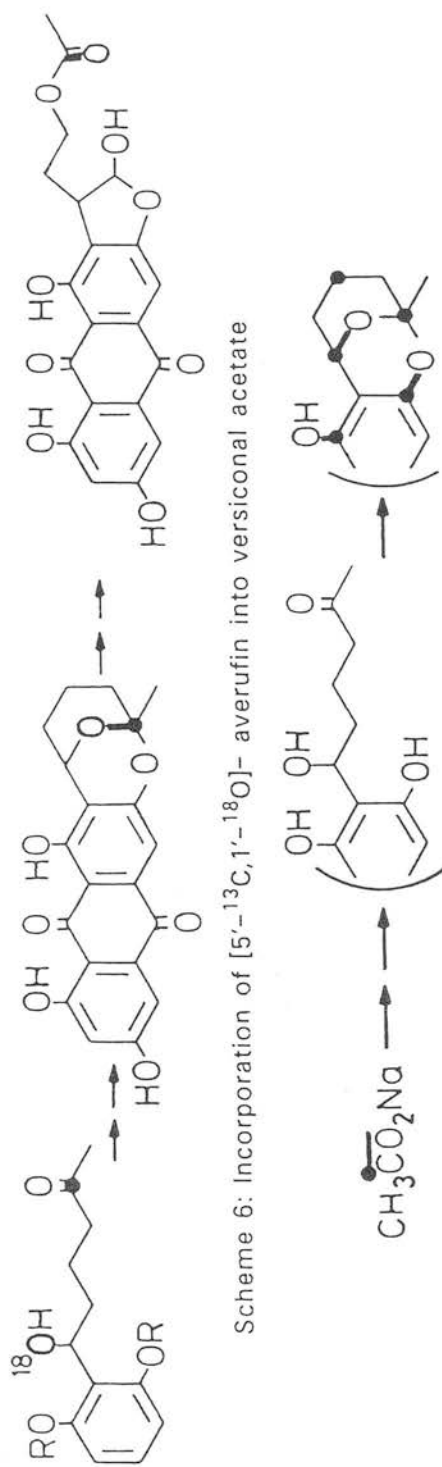
From early studies it became apparent that the contiguous 2- and 2'-carbons of versicolorin A (10) are both derived from the methyl carbon of acetate and thus a "1,2-aryl shift" has occurred. A range of mechanisms have been proposed, as shown in scheme 3. In 1976 Kingston proposed a pinacol rearrangement following activation of the C-2' of the open-chain form of averufin (8a)<sup>69</sup>. Tanabe suggested an open-chain Favorskii rearrangement of the C-1'-oxo compound (18)<sup>70</sup>. Steyn (1977) favoured initial dehydration and epoxidation of the open-chain form of averufin (8a) followed by rearrangement to the aldehyde (19). In 1982 Sankawa proposed a ring-closed pinacol rearrangement via nidurufin (20)<sup>64</sup>, a known *Aspergillus* metabolite<sup>71</sup>.

In 1981 Townsend and coworkers carried out an elegant synthesis of averufin (8), as outlined in scheme 4<sup>72</sup>, and used this methodology to produce a series of specifically labelled compounds. [4'-<sup>13</sup>C]- And [1'-<sup>2</sup>H<sub>1</sub>,1'-<sup>13</sup>C]-averufin were each specifically incorporated into both versicolorin A (10)<sup>73</sup> and aflatoxin B<sub>1</sub> (1)<sup>66</sup> (scheme 5). Thus the inner and outer acetate units in the side-chain of (10) (and(1)) must be derived respectively from the inner and central units of the side-chain of (8). Also, the retention of integrity of the 1'-(C-H) bond limits the oxidation level of that carbon to aldehyde, thus ruling out the possibility of a Favorskii rearrangement, which would require the formation of a carboxylate species. The other mechanisms in scheme 3 are consistent with the above result. Townsend proposed a further ring-closed pinacol rearrangement, similar to that of Sankawa (scheme 3).

All mechanisms in scheme 3 necessitate functionalisation at C-2' of averufin providing a suitable leaving group to be displaced by the incoming anthraquinone nucleus. This led to the proposed implication of nidurufin (20) in the biosynthesis of versiconal acetate. Nidurufin, first isolated from *A. nidulans*



Scheme 5: Incorporation of  $[4'-^{13}\text{C}]$ - and  $[1'-^2\text{H}, 1'-^{13}\text{C}]$ - averufins into versicolorin A and aflatoxin B<sub>1</sub>



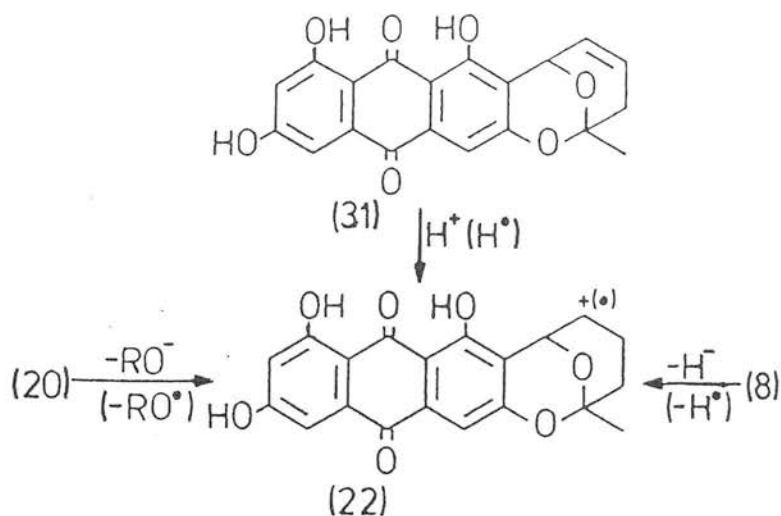
Scheme 6: Incorporation of  $[5'-^{13}\text{C}, 1'-^{18}\text{O}]$ - averufin into versiconal acetate

Scheme 7: Incorporation of sodium  $[1'-^{13}\text{C}, 1'^{18}\text{O}_2]$ - acetate into averufin (8)

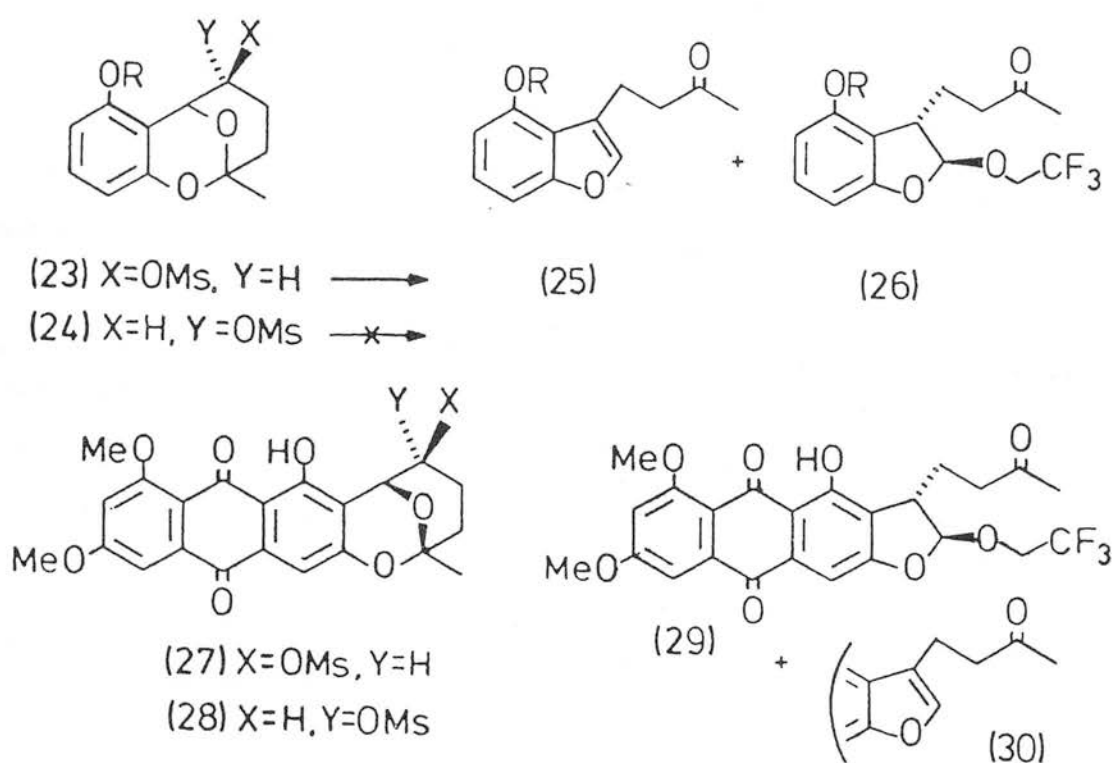
in 1970, bears a 2'-hydroxyl group. The *relative* stereochemistry of (20) was originally assigned as *endo* (21) on the basis of its  $^1\text{H}$ -n.m.r. vicinal proton coupling constant. However Townsend, using a modification of his averufin strategy, synthesised both *endo* (21) and *exo* (20) alcohols; comparison with a sample of the natural product showed that nidurufin has identical spectral characteristics to that of the *exo* epimer (20)<sup>66</sup>. In 1984 Cava synthesised the 6,8-dideoxy analogues of these *exo* and *endo* alcohols, and he too favoured the *exo* structure for nidurufin<sup>74</sup>. Only this epimer has the anthraquinone ring and the 2'-hydroxyl in the antiperiplanar disposition required for such a 1,2-shift. Cava suggested a further mechanism (scheme 3), similar to those of Sankawa and Townsend.

In 1985 Townsend and Koreeda determined the absolute stereochemistry of averufin as (1'-S)<sup>75</sup>, as indicated (8) in scheme 4, by the exciton chirality circular dichroism method<sup>76</sup>. The absolute stereochemistry of nidurufin is not known, but it is logical to assume that it also has the (1'-S) configuration as shown for (20).

From earlier precedent<sup>77</sup>, and latterly from model studies by Cava in 1983<sup>78</sup>, it has been shown that 1,2-aryl shifts are feasible *in vitro*. Townsend's group synthesised [1'- $^2\text{H}_1$ ]-nidurufin and its 2'-epimer, but neither gave any detectable incorporation into aflatoxin B<sub>1</sub> by *A. parasiticus*. [1'- $^{18}\text{O}$ , 5'- $^{13}\text{C}$ ]-Averufin was prepared and incorporated specifically into versiconal acetate with retention of integrity of the 5'-(C-O) bond, as determined by the  $^{18}\text{O}$ -induced shift in the C-5'  $^{13}\text{C}$  NMR signal (scheme 6)<sup>79</sup>. Thus *given* that ketalisation of the open-chain form (8a) of averufin follows the accepted mechanism, an assumption borne out by the [1- $^{13}\text{C}$ ,  $^{18}\text{O}_2$ ]-acetate labelling pattern of averufin (scheme 7)<sup>80</sup>, all of the open-chain rearrangements shown in scheme 2 may be ruled out as they would necessitate cleavage of



Scheme 8: Possible routes to proposed cation (or radical) initiating 1,2-aryl shift reaction

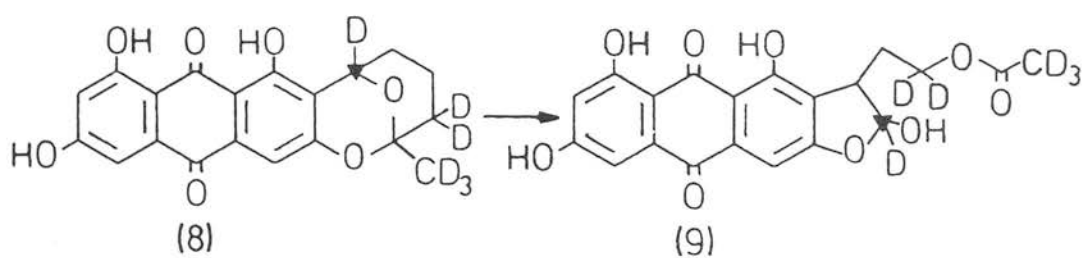
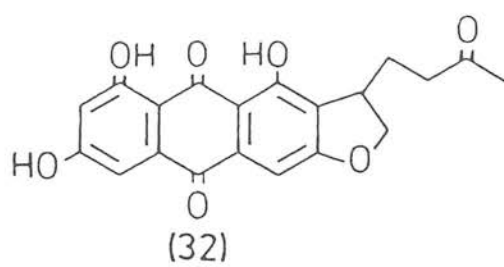


Scheme 9: Biomimetic 1,2-aryl shift reactions

the 5'-(C-O) bond.

The two results discussed in the preceeding paragraph indicate a ring-closed 1,2-aryl shift involving oxidation, but without hydroxylation, at C-2' of averufin, to perhaps a radical or cation, e.g. (22) (scheme 8). Townsend reported the synthesis of compounds (23) and (24) ( $R=CH_3OCH_2-$ ) (scheme 9). Refluxing (23) in trifluoroethanol resulted in rapid formation of (25;  $R=CH_3OCH_2-$ ). In buffered solution (26;  $R=CH_3OCH_2-$  and  $R=H$ ) were produced. In contrast, (24) remained unreactive under the same reaction conditions. The mesylates of dimethoxynidurufin (27) and its epimer (28) were synthesised to give a closer analogy to the *in vivo* reaction. Refluxing (27) in trifluoroethanol gave a mixture of (29) and (30), but it reacted more slowly than (23) did under the same conditions, suggesting that the presence of the electron-withdrawing anthraquinone moiety reduces its migratory aptitude. The *endo* epimer (28), as for (24), did not undergo solvolysis. This reluctance to rearrange reinforces the belief that for the *exo* compounds, the migrating and leaving groups have a mutually favourable stereoelectronic orientation, not present in the *endo* compounds<sup>81</sup>.

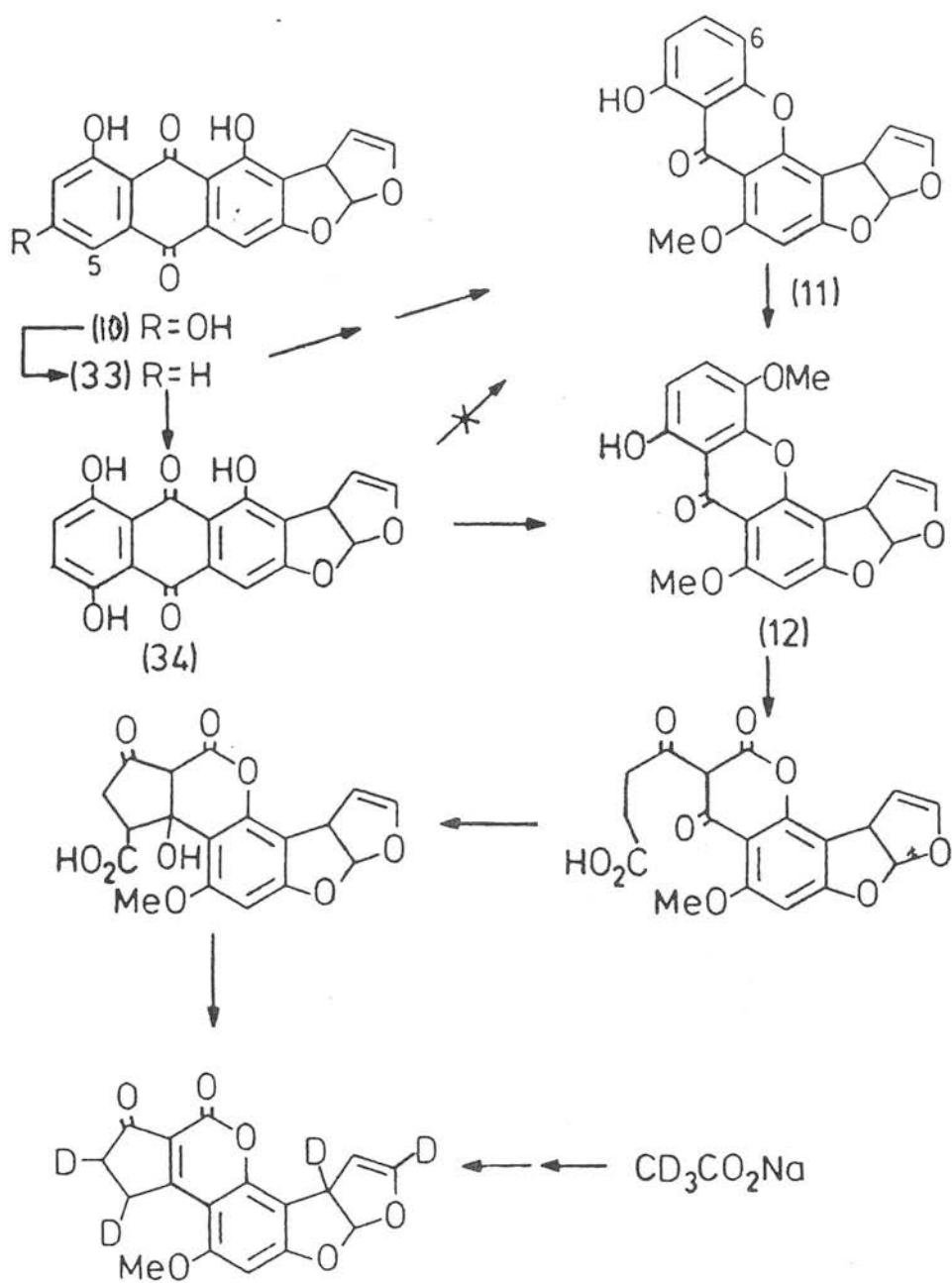
The proposed cation (or radical) intermediate (22) may derive from dehydroaverufin (31), by electrophilic attack by a proton (or by a hydrogen atom respectively). Although (31) is an *A. versicolor* metabolite<sup>82</sup>, its intermediacy in the above scheme has not hitherto been proposed in print. A further source of (22) is averufin itself, by an NADP-mediated hydride abstraction. Such a process, followed by a 1,2-shift has been observed in ring contraction during gibberellin biosynthesis<sup>83</sup>.



Scheme 10: Incorporation of [1',4',6'-<sup>2</sup>H<sub>6</sub>,1'-<sup>13</sup>C]-averufin into versiconal acetate

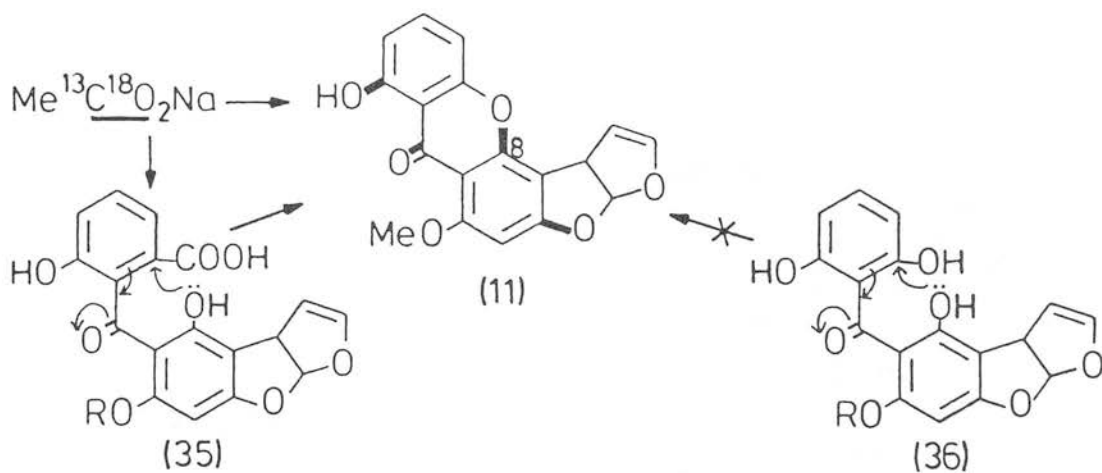
### 3.1.4. Oxygen Insertion into the Anthraquinone Side-chain

Inspection of any mechanism in scheme 3 implies that the insertion of oxygen between C-4' and C-5' of the averufin side-chain occurs after the 1,2-aryl shift. This belief is supported by the existence of versicolorone (32), an *A. versicolor* metabolite<sup>84</sup>. *A. parasiticus* treated with dichlorvos incorporated [1',4',6',-<sup>2</sup>H<sub>6</sub>,1'-<sup>13</sup>C]-averufin *intact* into versiconal acetate (9), as determined by deuterium n.m.r. spectroscopy and mass spectroscopy (scheme 10)<sup>85</sup>. This shows that formation of (9) occurs intramolecularly, rather than by degradation of the side-chain followed by trivial acetylation. This, and intact incorporation of [5'-<sup>13</sup>C,1'-<sup>18</sup>O]-averufin into versiconal acetate (see previous section), provide strong evidence for a biological Baeyer-Villiger reaction.

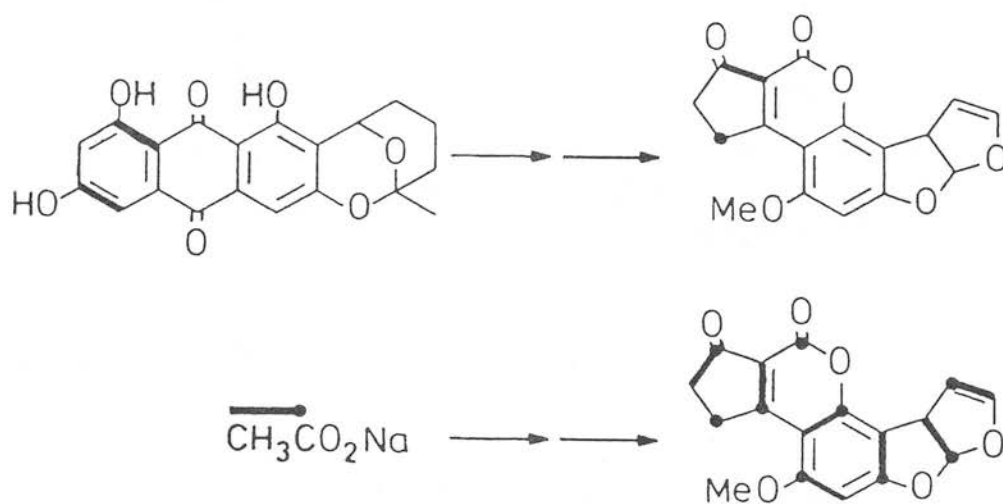


Scheme 11: Postulated late stages in aflatoxin biosynthesis





Scheme 12: Incorporation of sodium  $[1-^{13}\text{C}, ^{18}\text{O}_2]$ -acetate into sterigmatocystin



Scheme 13: Incorporation of a mixture of  $[5,6-^{13}\text{C}_2]$ - and  $[8,11-^{13}\text{C}_2]$ -averufins into aflatoxin  $\text{B}_1$

### 3.1.5. Xanthone Formation and Rearrangement

Given that the C-6 hydroxyl of versicolorin A (10) is no longer present in sterigmatocystin (11), and that (12), (33) and (34)<sup>86</sup> are known natural products, two plausible routes may be envisaged (scheme 11). Simpson and coworkers have incorporated  $[2-^2\text{H}_3]-$  and  $[2-^2\text{H}_3, 1-^{13}\text{C}]-$ acetates into sterigmatocystin (11)<sup>87</sup>. The retention of acetate-derived hydrogen at C-6 shows that (34) cannot be a precursor to (11), so that in the conversion of anthraquinone to xanthone, no mechanism which involves a phenolic hydroxyl or a carbonyl at C-6 is feasible.

Vederas has incorporated  $[1-^{13}\text{C}, ^{18}\text{O}_2]-$ <sup>acetate</sup> into sterigmatocystin as shown in scheme 12<sup>88</sup>. Retention of integrity of the 8-(C-O) bond shows that xanthone formation may occur via (35). The intermediacy of (36) is disfavoured because a symmetrical portion of a molecule would show randomisation of  $[1,2-^{13}\text{C}_2]-$ acetate label, *unless* this portion were tightly enzyme-bound to prevent free rotation.

The incorporation of  $[2-^2\text{H}_3]-$ acetate into aflatoxin B<sub>1</sub><sup>89</sup> (scheme 11) results in the presence of deuterium at a carboxyl derived carbon, suggesting intramolecular migration from a methyl derived carbon. The most likely explanation for this is a NIH-shift<sup>90</sup> resulting from hydroxylation of either (11) (at C-6), or (33) (at C-5). Ring cleavage of (12) followed by an intramolecular aldol condensation, decarboxylation and dehydration would lead to formation of aflatoxin B<sub>1</sub> with the observed labelling pattern (scheme 11).

In 1983 Townsend incorporated an equal mixture of  $[5,6-^{13}\text{C}_2]-$  and  $[8,11-^{13}\text{C}_2]-$  averufin into aflatoxin B<sub>1</sub> (1)<sup>91</sup>, as shown in scheme 13. Carbons 2, 3 and 5 of (1) are labelled, with the 2-3 bond intact. Comparison of this pattern with the labelling pattern of aflatoxin B<sub>1</sub> derived from  $[^{13}\text{C}_2]-$ acetate<sup>60</sup> indicates

that carbons 6, 8 and 11 of averufin correspond respectively to carbons 5, 3 and 2 of aflatoxin B<sub>1</sub>, and that C-5 of averufin is lost. This result is consistent with the xanthone rearrangement shown in scheme 11.

## 3.2. Discussion

### 3.2.1. Summary

The work undertaken in this chapter is summarised as follows: synthesis and incorporation of  $[2-^2\text{H}_2]$ -hexanoate and  $[2-^{13}\text{C}]$ -malonate into averufin, attempted manipulation of the averufin side-chain, and synthesis of deuteriated averufin, and attempted incorporation into sterigmatocystin and aflatoxin B<sub>1</sub>.

### 3.2.2. Early stages of Averufin biosynthesis

It was decided to confirm Townsend's incorporation of hexanoate into averufin by feeding deuterium-labelled hexanoate. Accordingly, ethyl hexanoate was subjected to exchange conditions (sodium methoxide in deuteriomethanol) which gave either methyl hexanoate or a mixture of methyl and ethyl hexanoates. These were hydrolysed to give the free acid; the triplet at 2.35 ppm in its  $^1\text{H}$ -n.m.r. spectrum, due to the 2-position, had diminished in intensity by about 80%; its deuterium nmr spectrum gave a singlet at 2.36 ppm. This material was administered to *A.parasiticus* under the same two feeding regimes as described by Townsend<sup>66</sup>. Averufin was isolated in both cases, and converted into its triacetate.

The  $^2\text{H}$  n.m.r. spectrum of the triacetate of averufin derived from *A.parasiticus* grown under shaken conditions showed two signals, at 1.57 and 1.98 ppm. These signals required a long acquisition time, and were broad, as expected from a deuterium spectrum. Also, the  $^1\text{H}$  n.m.r. spectrum of averufin has not been fully assigned with respect to the six methylene protons. However it was clear that hexanoate has been incorporated into averufin without noticeable degradation to acetate, by comparison of this spectrum with that of the triacetate of averufin derived from  $[2-^2\text{H}_3]$ -acetate. The latter shows different signals at high field (including an intense signal due to the starter

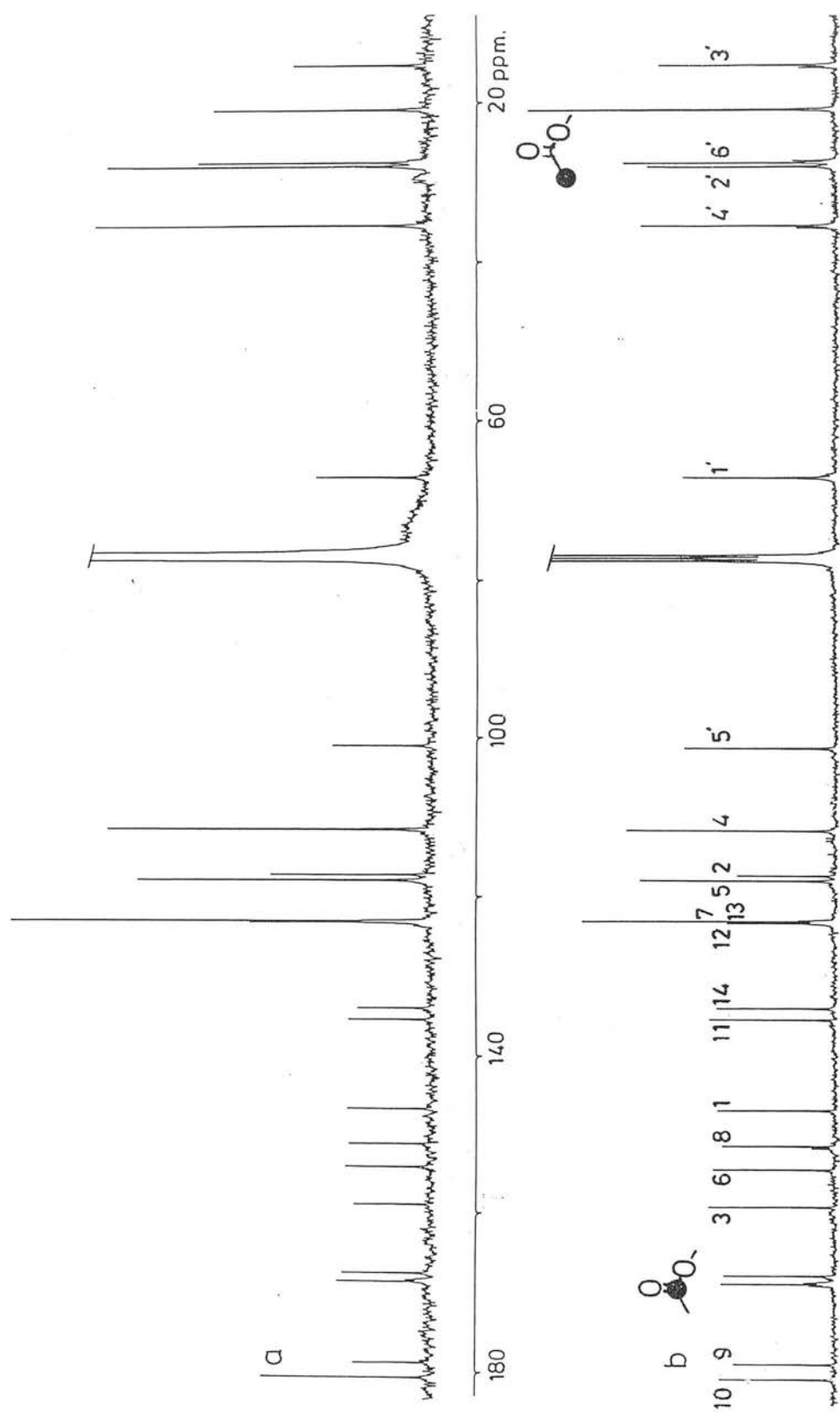


Figure 1: 91 MHz.  $^{13}\text{C}$  N.M.R. spectra of averufin labelled from diethyl [2- $^{13}\text{C}$ ]- malonate.  
 1a: Spectrum of labelled averufin diacetate;  
 1b: Spectrum of unlabelled averufin diacetate.

Carbon	$\delta(\text{ppm})$	A	B	A/B	(A/B)*
C-3'	15.53	4.382	5.522	0.794	1.0
COCH <sub>3</sub>	20.92	6.947	9.633	0.721	0.9
6'	27.58	7.428	6.639	1.119	1.4
2'	28.10	10.320	5.891	1.752	2.2
4'	35.52	10.709	6.080	1.761	2.2
1'	67.28	3.672	4.772	0.769	1.0
5'	101.11	3.171	4.723	0.671	0.8
4	111.66	10.317	6.539	1.578	2.0
2	117.44	5.105	3.067	1.664	2.1
5	118.02	9.336	6.113	1.527	1.9
7+13	123.26	13.377	7.913	1.690	2.1
12	123.41	5.763	3.377	1.707	2.1
14	134.15	2.359	3.702	0.637	0.8
11	135.61	2.641	3.929	0.672	0.8
1	146.94	2.693	3.671	0.734	0.9
8	151.36	2.636	3.529	0.747	0.9
6	154.33	2.766	3.801	0.728	0.9
3	159.14	2.494	3.951	0.631	0.8
COCH <sub>3</sub>	167.71	2.852	3.474	0.821	1.0
COCH <sub>3</sub>	168.76	3.014	3.557	0.847	1.1
9	179.01	2.497	3.167	0.778	1.0
10	180.92	5.422	3.619	1.498	1.9

Table 1: Comparison of signal intensities in the 91MHz.

<sup>13</sup>C n.m.r. spectra of (A) averufin diacetate  
labelled from diethyl [2-<sup>13</sup>C]-malonate and  
(B) unlabelled averufin diacetate.

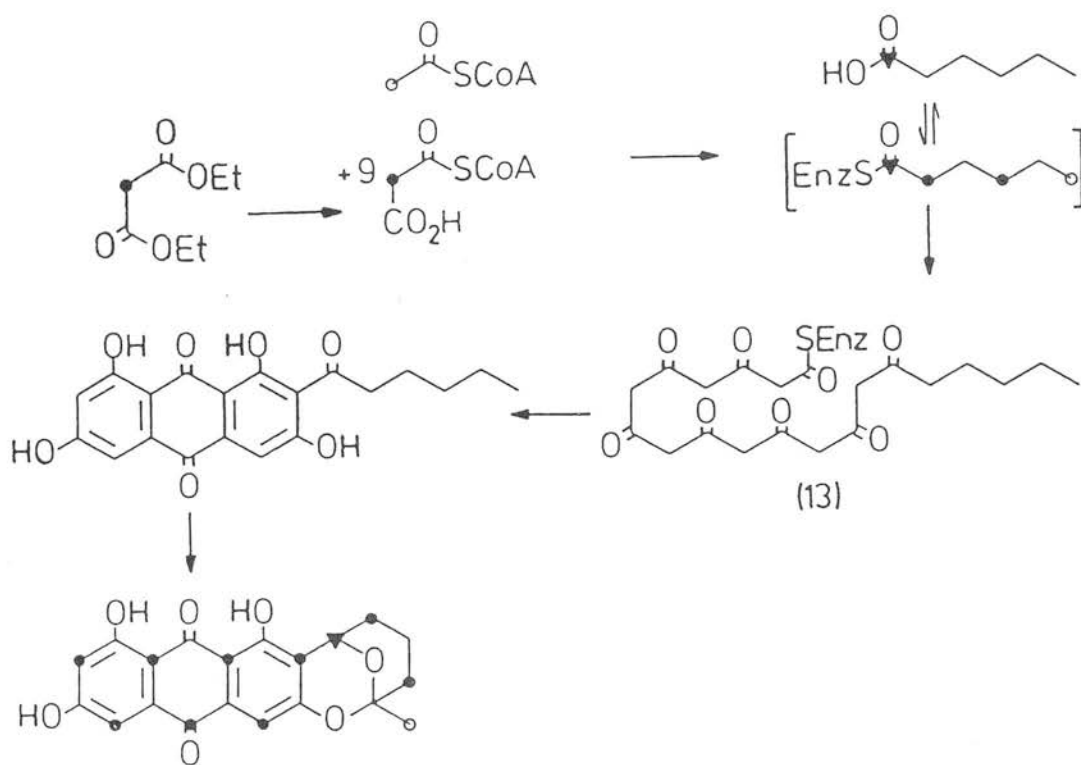
\* In the final column, each signal ratio of A to B is normalised  
to make the average of the 4 acetate ratios equal to 1.0.

methyl group), and also low field signals due to the 4 and 5 (but not the 7) aromatic positions. Hence this result is in agreement with Townsend's specific incorporation of [1- $^{13}\text{C}$ ]-hexanoate into averufin.

The triacetate of averufin derived from *A.parasiticus* grown under static conditions gave the same result, but the n.m.r. signals obtained were weaker.

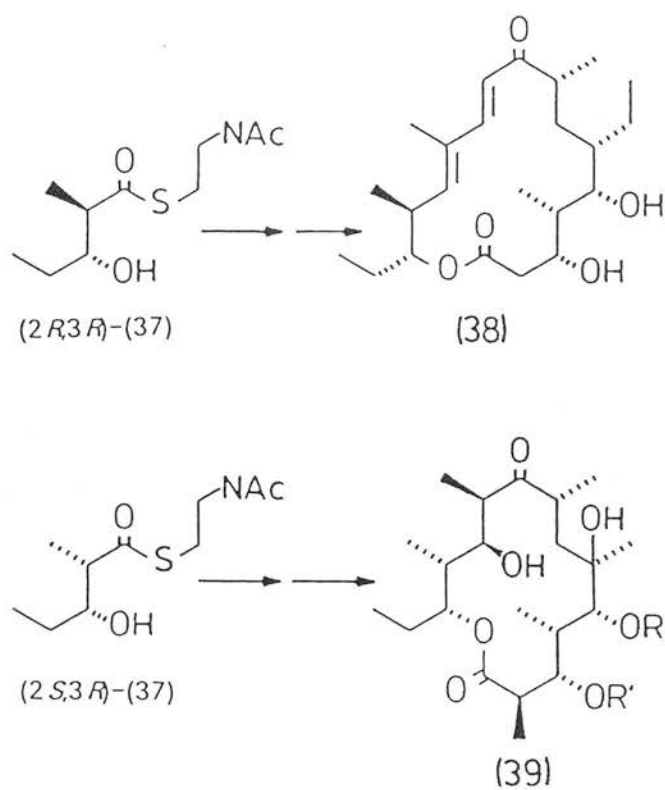
As mentioned in section 3.1, intact incorporation of hexanoate can be interpreted in two ways; either hexanoate is independently synthesised and then used as a starter unit for averufin biosynthesis, or it is produced as an intermediate by the polyketide synthase, but can equilibrate with free hexanoyl CoA at this stage. It was hoped that the incorporation of [2- $^{13}\text{C}$ ]-malonate into averufin would distinguish between these two possibilities. If hexanoate is independently synthesised, then malonate incorporation should be uniform for the anthraquinone portion of averufin, but lower in the  $\text{C}_6$  side-chain. If averufin is a true decaketide, then incorporation of malonate should be uniform for nine positions, with lower incorporation into the starter unit.

Accordingly, diethyl [2- $^{13}\text{C}$ ]-malonate was synthesised as described in chapter 2<sup>92,93</sup>, and administered to a static culture of *A.parasiticus* ATCC 22451. Averufin was subsequently isolated as described previously, and conversion to its triacetate was attempted. However the major product obtained was averufin diacetate, by n.m.r. and mass spectroscopy. Its  $^{13}\text{C}$  nmr spectrum was obtained under conditions of inverse gated decoupling (in order to suppress n.O.e. effects), and in the presence of the paramagnetic relaxation reagent chromium tris-acetoacetate, in order to normalise all signal intensities. The spectrum was compared with one obtained from "natural abundance" averufin diacetate run under the same conditions (see Figure 1). The intensity of each signal for the unlabelled species was divided by the intensity of the corresponding signal



Scheme 14: Incorporation of diethyl [2-<sup>13</sup>C]- malonate into averufin



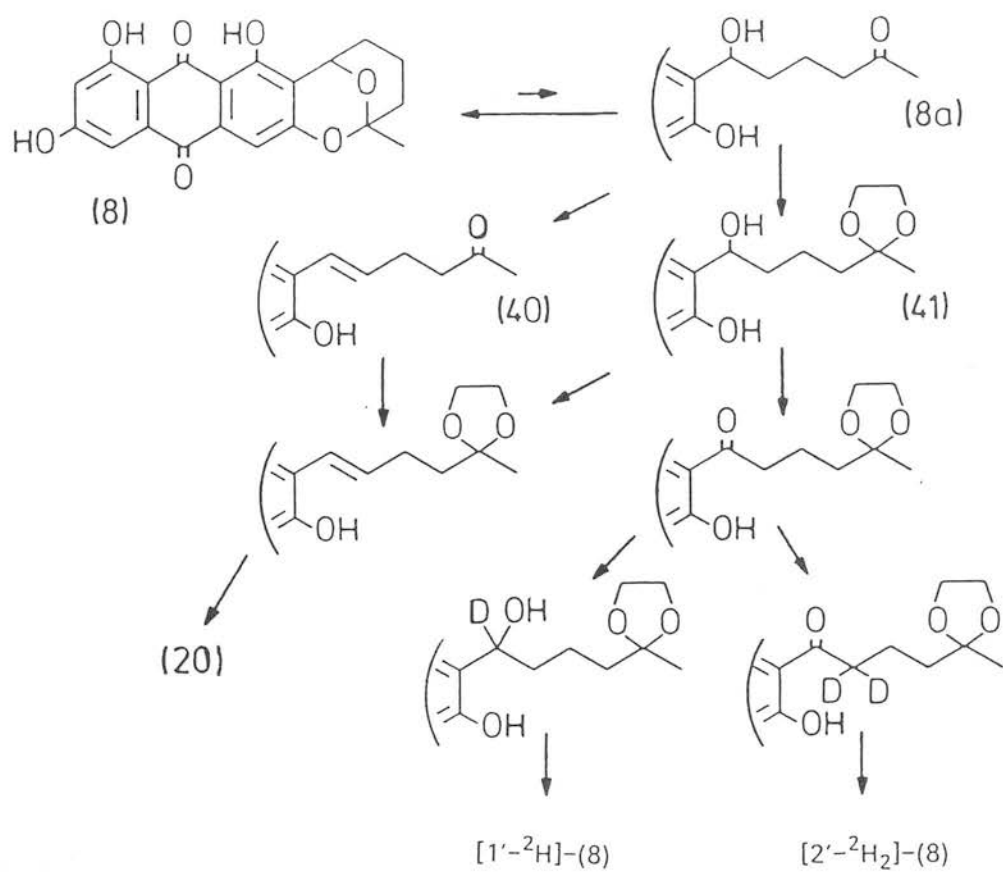


Scheme 15: Incorporation of labelled 2-methyl-3-hydroxy-pentanoates into tylactone (38) and erythromycin (39)

for the natural abundance sample. These values were normalised by making the average of the O-acetyl values equal to 1.00. Scrutiny of these values (see Table 1) indicated that enrichment had occurred at those positions known to be derived from the methyl carbon of acetate<sup>94</sup>. The enrichment was essentially equal for nine out of ten of these positions, but lower at the C-6' position, the position known to be derived from the methyl carbon of the *starter* acetate unit<sup>65</sup>.

Thus a starter effect appears to be operating, which suggests that averufin is a decaketide, formed by successive additions of nine malonate units (with loss of carbon dioxide) to the starter acetate unit. It would appear that the addition of the first and second malonate units are each followed by reduction, elimination and hydrogenation, analogous to fatty acid biosynthesis, to form enzyme-bound hexanoate. Chain assembly continues with the remaining seven malonate units adding successively, but without the intervening fatty acid synthesis steps. Thus an intermediate such as (13) is probably formed, and cyclises to norsolorinic acid (the first isolated intermediate in this pathway) which is further elaborated to averufin (Scheme 14).

The observation that hexanoate is incorporated intact into averufin suggests that the hexanoate formed on the polyketide synthase is free to equilibrate with exogenous hexanoyl CoA. It has been speculated that intermediates more advanced than hexanoate may be incorporated intact provided they can equilibrate with the enzyme-bound intermediate. Indeed, at the time of writing two important results reported by Hutchison and Cane show that the N-acetyl-cysteamine thioester of (2*R*, 3*R*)-2-methyl-3-hydroxy-pentanoate (37) was incorporated intact into tylactone (38)<sup>95</sup>, and its (2*S*, 3*R*)-epimer was incorporated intact into erythromycin (39) (scheme 15)<sup>96</sup>. These results point towards a potentially very fruitful area of study in the biosynthesis of



Scheme 16: Proposed manipulation of the averufin side-chain

polyketides. However it is pertinent to repeat here the inability to incorporate (intact) 3-oxo-octanoate (17) (which would logically appear to be an intermediate) into averufin<sup>68</sup>. This may indeed be an intermediate, but is unable to leave the enzyme surface at this stage. Also, as a  $\beta$ -dicarbonyl compound, (17) may be reactive under basic conditions, or may degrade to acetate (and perhaps other compounds) before reaching the synthetase enzyme.

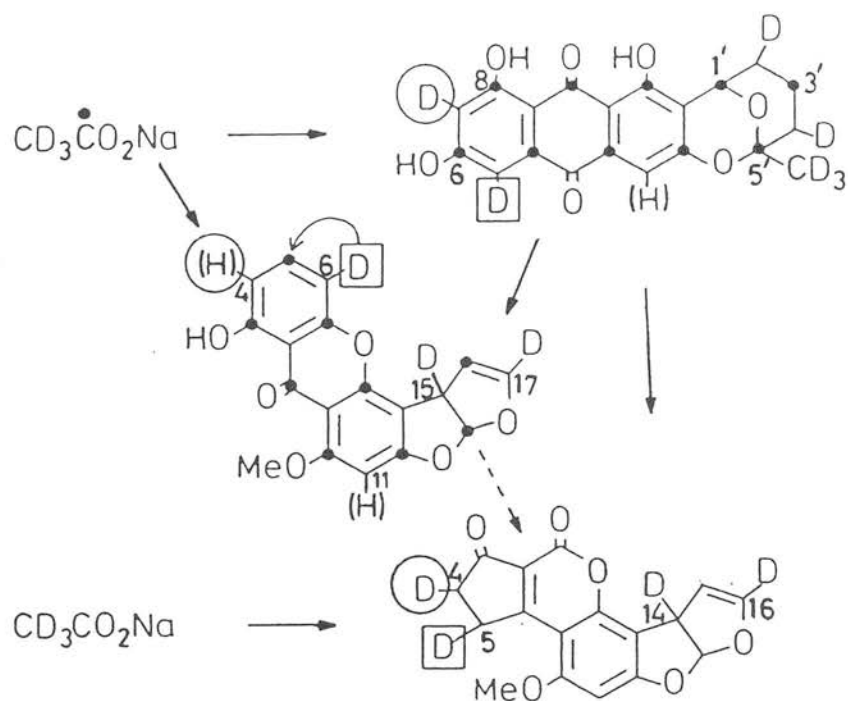
### 3.2.3. Attempted Manipulation of the Averufin Side-Chain

In order to gain an insight into the mechanism operating during the 1,2-aryl shift converting averufin to versiconal acetate, it was intended to synthesise averufin specifically labelled at various positions in the ketal side-chain (see scheme 16). In addition, it was reasoned that labelled nidurufin could also be synthesised, with a view to checking its intermediacy in aflatoxin biosynthesis.

Dehydration of the open-chain (keto) (8a) form of averufin, across the 1' and 2' positions to give (40), was attempted. This however was not achieved; even the use of concentrated acid returned averufin as the only identifiable product. This acid stability has previously been reported<sup>24</sup>.

The use of base to dehydrate (8) was also unsuccessful; small amounts of a red compound, of higher polarity than averufin, were isolated. This may have been 1,3,6,8-tetrahydroxyanthraquinone, from the simplicity of the <sup>1</sup>H n.m.r. spectrum of its peracetate, but the compound was not further characterised.

It was hoped that manipulation of the averufin side-chain would be easier if the open-chain (keto) form (8a) could be "trapped", and thus its ethane-1,2-diol adduct (41) was considered as a useful derivative. However the treatment of averufin with ethane-1,2-diol under standard conditions failed to give the expected product. Evidence for the existence of the ketonic form (8a) of averufin comes from its structure determination<sup>24</sup>; it was shown to undergo



Scheme 17: Incorporation of deuterium-labelled acetates into averufin, sterigmatocystin and aflatoxin B<sub>1</sub>

acid-catalysed deuterium exchange, with an increase in molecular weight of five atomic mass units, as determined by mass spectrometry. Thus given that such an equilibrium between (8) and (8a) does exist, it must be far to the left. From an entropic viewpoint this is reasonable, as formation of the ketone (8a) would require attack by two water molecules.

### 3.2.4. Incorporation of Specifically Deuteriated Averufin into Aflatoxin B<sub>1</sub>

The incorporation by Simpson of deuterium labelled acetates into averufin, sterigmatocystin and aflatoxin B<sub>1</sub> is shown in scheme 17. In the <sup>13</sup>C nmr spectrum of [1-<sup>13</sup>C,2-<sup>2</sup>H<sub>3</sub>]-acetate-enriched averufin, β-shifted signals were observed for positions corresponding to carbons 6, 8, 1', 3' and 5', indicating the presence of deuterium at carbons 5, 7, 2', 4' and 6' respectively. Less deuterium was incorporated at C-7 than C-5, and none was retained at C-4<sup>65</sup>. Sankawa reported the incorporation of deuterium from [1-<sup>13</sup>C,<sup>2</sup>H<sub>3</sub>]-acetate into averufin at the positions mentioned above, plus also at the 4-position<sup>64</sup>. However incorporation was determined by the relative decrease in the doublet due to <sup>13</sup>C coupled to one proton. Incorporation of [1-<sup>13</sup>C,2-<sup>2</sup>H<sub>3</sub>]-acetate into sterigmatocystin shows that deuterium is present at carbons 6, 15 and 17 (corresponding respectively to the 5, 2' and 4' positions of averufin)<sup>87</sup>. There was no evidence for incorporation of deuterium into the C-4 or C-11 positions, both of which were reported by Sankawa. Incorporation of [2-<sup>2</sup>H<sub>3</sub>]-acetate into aflatoxin B<sub>1</sub> shows that deuterium is present at carbons 4, 5, 14 and 16, corresponding respectively to the 4, 5, 15 and 17 positions of sterigmatocystin<sup>89</sup>. This anomaly could be clarified by the specific deuterium labelling of averufin at the 7-position followed by its incorporation into sterigmatocystin and aflatoxin B<sub>1</sub>. It was expected that the 7-position of averufin would undergo base-catalysed deuterium exchange more rapidly than

the other positions. This was confirmed by subjecting a small sample to such conditions (sodium [ $^2\text{H}_3$ ]-methoxide in [ $^2\text{H}_4$ ]-methanol), and monitoring any changes in n.m.r. signal intensity.

Exchange occurred first at the 7-position, then more slowly at the 5-position, by which time the molecule started to degrade. Thus [7- $^2\text{H}$ ]-averufin was synthesised by stirring averufin in a solution of sodium methoxide in [ $^2\text{H}_1$ ]-methanol. It was decided to use averufin labelled in the side chain as an internal standard, as its incorporation into aflatoxin  $\text{B}_1$  has already been studied. [4',6'- $^2\text{H}_5$ ]-Averufin was synthesised by acid catalysed exchange, using established methods<sup>24</sup>.

In order to feed labelled averufin to sterigmatocystin and to aflatoxin  $\text{B}_1$ , it was necessary to construct a growth-production curve for each metabolite in order to determine the optimum feeding time. Thus a crude extract was obtained from *A. parasiticus* every 24 hours for ten days, and its sterigmatocystin content monitored by high performance liquid chromatography.

The first solvent system used gave a peak with the same retention time as a standard sample of sterigmatocystin, but use of a second solvent system failed to show such a correlation. The absence of sterigmatocystin was confirmed by analytical thin layer chromatography of a crude extract from five days growth against a standard sample of sterigmatocystin. It is possible that the fungal strain failed to produce sterigmatocystin as a result of repeated subculturing.

In a similar manner, crude extracts were obtained from *A. flavus* every 24 hours for ten days, and a peak with the same retention time as a standard sample of aflatoxin  $\text{B}_1$ , using a range of solvent programmes, was evident.

Thus a mixture of [7-<sup>2</sup>H]-averufin and [4',6'-<sup>2</sup>H<sub>5</sub>]-averufin was administered to growing mycelia of *A.flavus* , 24, 36, 48 and 60 hours after inoculation. A further 24 hours later, a crude extract was obtained, and from this was isolated a very small amount of aflatoxin B<sub>1</sub>. Its deuterium n.m.r. spectrum showed signals at 2.57 and 6.67 ppm., corresponding respectively to the 4 and 16 positions of aflatoxin B<sub>1</sub>. However signals were also observed at 1.54, 3.63 and 4.12 ppm. The small amount of material available precluded further studies to clarify the labelling pattern. Further studies are required to obtain more definitive evidence.



### 3.3. Experimental

For general procedures see section 2.3. Constituents of fungal growth media are listed below:

#### Blakeslees malt extract agar

dextrose	2.0% w/v
malt extract	2.0%w/v
bacto agar	2.0% w/v
bacto peptone	2.0% w/v
sodium chloride	0.05% w/v
distilled water	to 100% w/v

#### Sucrose-asparagine-ammonium sulphate medium (Low salts medium)

sucrose	8.5% w/v
asparagine	1.0% w/v
ammonium sulphate	0.35% w/v
potassium orthophosphate	0.075% w/v
magnesium sulphate heptahydrate	0.035% w/v
calcium chloride dihydrate	0.0075% w/v
zinc sulphate heptahydrate	0.001% w/v
manganese(IV) chloride tetrahydrate	0.0005% w/v

ammonium molybdate(VI) tetrahydrate	0.0002% w/v
sodium tetraborate heptahydrate	0.0002% w/v
iron(II) sulphate heptahydrate	0.0002% w/v
doubly distilled water	to 100%.

#### Minimal mineral medium

glucose	5.0% w/v
ammonium sulphate	0.3% w/v
potassium orthophosphate	1.0% w/v
magnesium sulphate heptahydrate	0.2% w/v
sodium tetraborate decahydrate	0.07% w/v
ammonium molybdate(VI) tetrahydrate	0.05% w/v
iron(III) sulphate hexahydrate	0.001% w/v
copper(II) sulphate pentahydrate	0.00003% w/v
manganese(II) sulphate monohydrate	0.000011% w/v
zinc sulphate heptahydrate	0.00176% w/v
distilled water	to 100%.

#### Low sugar replacement medium

glucose	1.5% w/v
potassium orthophosphate	0.5% w/v
magnesium sulphate heptahydrate	0.05% w/v
potassium chloride	0.05% w/v

sodium tetraborate decahydrate	0.07% w/v
ammonium molybdate(VI) tetrahydrate	0.05% w/v
iron(III) sulphate hexahydrate	0.001% w/v
copper(II) sulphate pentahydrate	0.00003% w/v
manganese(II) sulphate monohydrate	0.000011% w/v
zinc sulphate heptahydrate	0.00176% w/v
distilled water	to 100%.

## Production and Isolation of Averufin

*Aspergillus parasiticus* ATCC 24451 was stored in darkness at 4°C under paraffin on slopes of Blakeslees malt extract agar.

A spore suspension in distilled water was inoculated into 500ml. Erlenmeyer flasks, or 2500ml. penicillin flasks, containing either 100ml. or 500ml. respectively of sucrose-asparagine-ammonium sulphate medium (low salts medium)<sup>98</sup>. The culture was incubated at 26°C in constant light. A mycelial mat began to form after two days, and its underside started to show orange pigmentation within three days. After twelve days the mycelia were drained and homogenised in chloroform-methanol (50:50) in a blender. The resultant green slurry was filtered, and the filtrate reduced *in vacuo* to give a cloudy brown solution. This was partitioned between methanol-water (90:10) and hexane; most colour remained in the polar layer, which was further partitioned between chloroform and water. The aqueous layer was extracted into water and the combined chloroform fractions were amalgamated, dried (magnesium sulphate) and reduced *in vacuo* to give a brown solid (approximately 200mg. per litre of medium). This was purified by column chromatography, using chloroform-methanol (97:3) as eluant. The major orange pigment, corresponding to an authentic sample of averufin by analytical thin layer chromatography, was isolated as an orange solid (approximately 120mg. per litre of medium). Recrystallisation from ethanol afforded orange needles, m.p. (lit.<sup>24</sup> 280–282°C).

– <sup>1</sup>H n.m.r. (60 MHz., (CD<sub>3</sub>)<sub>2</sub>SO<sub>2</sub>-CDCl<sub>3</sub>): 1.52 (3H, s, 6'-CH<sub>3</sub>), 1.3–2.1 (6H, m, 3',4',5'-(CH<sub>2</sub>)<sub>3</sub>), 5.16 (1H, br m, 1'-CH), 6.38 (1H, d, J=2.1Hz., 7-CH), 6.86 (1H, s, 4-CH), 6.97 (1H, d, J=2.1Hz., 5-CH), 11.78 (s, exch, ArOH), 12.15 (s, exch, ArOH) ppm.

*[2-<sup>2</sup>H<sub>2</sub>]-Hexanoic acid*

Dry sodium (400mg.,17.4mmoles) was cleaned and dissolved in cooled deuteriomethanol (10ml.). Ethyl hexanoate (3.5g.,24.3mmoles) was added. After stirring for 16 hours with exclusion of moisture, the product was acidified (2N hydrochloric acid) and reduced *in vacuo* to give a clear liquid. This was partitioned between water and chloroform; the water was washed with ether. The combined organic extracts were reduced *in vacuo* to give a pale yellow liquid, a mixture of deuterated methyl and ethyl hexanoates (1.425g.). These were hydrolysed by stirring in boiling 2N sodium hydroxide solution for 2.5 hours. The product was acidified (2N hydrochloric acid) and extracted into ether, which was dried and reduced *in vacuo* to give a yellow liquid, *[2-<sup>2</sup>H<sub>2</sub>]-hexanoic acid*.

- <sup>1</sup>H N.M.R. (60 MHz., CDCl<sub>3</sub>): 0.90 (3H, t, J=5Hz., CH<sub>2</sub>CH<sub>3</sub>),  
1.3-1.8 (6H, m, 3,4,5-(CH<sub>2</sub>)<sub>3</sub>), 2.35 (t, residual 2-CH<sub>2</sub>+ CHD, 9.75  
(1H, brs, exch, CO<sub>2</sub>H).
- <sup>2</sup>H N.M.R. (31 MHz.,CHCl<sub>3</sub>): 2.36ppm.

**Incorporation of [2-<sup>2</sup>H<sub>2</sub>]-Hexanoic acid into Averufin**

**A: Shaken culture**

A spore suspension of *A.parasiticus* ATCC 22451 was inoculated into eight 500ml. Erlenmeyer flasks, each containing 100ml. of the minimal mineral medium of Adye and Mateles<sup>99</sup>. The seed culture was incubated at 26°C in constant light on an orbital shaker. After three days the spherical mycelial pellets started to turn orange. The flasks' contents were filtered through cheesecloth to give a wet weight of 110g. of mycelia. This was inoculated into

eleven 500ml. Erlenmeyer flasks each containing 100ml. of the low sugar replacement medium of Adye and Mateles. [2-<sup>2</sup>H<sub>2</sub>]-hexanoic acid (125mg.) was added to a portion of the above medium (22ml.) to give a slightly cloudy solution, which was distributed equally among the eleven flasks.

After incubation for 24 hours at 26°C in constant light on an orbital shaker, the flasks' contents were filtered, dried and extracted continuously for four hours with hexane, then for sixteen hours with acetone. The yellow solution was reduced *in vacuo*, and partitioned between chloroform and water. The organic layer was dried and reduced *in vacuo* to yield an orange solid. This was purified by preparative thin layer chromatography (chloroform-methanol (96:4)). The main band (R<sub>f</sub> 0.4) was isolated as an orange solid, 124mg., corresponding by analytical thin layer chromatography to an authentic sample of averufin.

#### B: Static Culture

A spore suspension of *A.parasiticus* 24451 was inoculated into four 500ml. Erlenmeyer flasks each containing 100ml. of low salts medium. The flasks were incubated at 26°C in constant light. Growth was slow, and the underside of the mycelia only began to turn orange after 80 hours. [2-<sup>2</sup>H<sub>2</sub>]-hexanoic acid (160mg.) was dissolved with difficulty in sterile distilled water (11ml.) and ethanol (0.5ml.). After 96 hours from incubation, 2ml. of the prepared feed was inoculated under the mycelial mat in each of two flasks. This was repeated 24 and 48 hours later for both flasks.

A further 24 hours later, the flasks' contents were extracted as described in the previous section, to yield an orange solid (289mg.), which was purified by column chromatography (chloroform-methanol (96:4)). The most intense band (R<sub>f</sub> 0.4 by analytical thin layer chromatography using the same eluant) was

isolated as an orange solid, 231mg., corresponding to an authentic sample of averufin.

#### Acetylation of Averufin derived from Shaken Hexanoate Feed

Averufin isolated from *A.parasiticus* fed with [2-<sup>2</sup>H<sub>2</sub>]-hexanoic acid (124mg.,0.37mmoles) was acetylated with acetic anhydride (2ml.) and pyridine as described above. The product, a yellow solid (73mg.), was purified by preparative thin layer chromatography (chloroform-methanol (98.5:1.5)) to give a yellow solid(R<sub>f</sub> 0.8) which was recrystallised from ethanol to give yellow needles, m.p. 203-206°C (lit.<sup>24</sup> 210-214°C).

- <sup>1</sup>H N.M.R. (60 MHz., CDCl<sub>3</sub>) : 1.53 (3H, s, 6'-CH<sub>3</sub>), 1.6-2.2 (6H, m, 3',4',5'-(CH<sub>2</sub>)<sub>3</sub>), 2.28 (3H, s, -COCH<sub>3</sub>), 2.35 (3H, s, -COCH<sub>3</sub>), 2.37 (3H, s, -COCH<sub>3</sub>), 5.10 (1H, br m, 1'-CH), 7.10 (1H, d, J=2.1Hz., 7-CH), 7.50 (1H, s, 4-CH), 7.81 (1H, d, J=2.1Hz., 5-CH) ppm.

- <sup>2</sup>H N.M.R. (55 MHz.,CHCl<sub>3</sub>, 50000 scans): 1.57, 1.98ppm.

#### Acetylation of Averufin derived from Static Hexanoate feed

Averufin isolated from *A.parasiticus* fed with [2-<sup>2</sup>H<sub>2</sub>]-hexanoic acid (231mg.,0.63mmoles), was stirred with acetic anhydride (4ml.) and pyridine (5 drops) at 100°C for 1.5 hours with the exclusion of moisture. The product was poured on ice, giving a yellow precipitate, which was taken up in chloroform and washed with 2N hydrochloric acid, then with water. The chloroform solution was dried and reduced *in vacuo* to yield a yellow solid. This was purified as above.

–  $^1\text{H}$  N.M.R. (60 MHz.,  $\text{CDCl}_3$ ) : 1.52 (3H, s, 6'- $\text{CH}_3$ ), 1.6–2.1 (6H, m, 3',4',5'-( $\text{CH}_2$ ) $_3$ ), 2.28 (3H, s, - $\text{OCOCH}_3$ ), 2.38 (6H, s, - $\text{OCOCH}_3$ ), 5.15 (1H, br m, 1'-CH), 7.16 (1H, d,  $J=2.1\text{Hz.}$ , 7-CH), 7.52 (1H, s, 4-CH), 7.82 (1H, d,  $J=2.1\text{Hz.}$ , 5-CH) ppm.

–  $^2\text{H}$  N.M.R. (55 MHz.,  $\text{CHCl}_3$ , 14000 scans): 1.55ppm.

### Incorporation of Diethyl [2- $^{13}\text{C}$ ]-Malonate into Averufin

A spore suspension of *A.parasiticus* ATCC 24451 was inoculated into four 500ml. Erlenmeyer flasks each containing 100ml. of low salts medium. The cultures were incubated at 26°C in constant light. Diethyl [2- $^{13}\text{C}$ ]-malonate (synthesised as described as described in chapter 2) (97mg.) was dissolved in ethanol (0.9ml.). This was added to one flask in three equal portions, at 48, 72 and 96 hours after inoculation.

A further 24 hours later the mycelia were filtered and oven-dried. The dried mycelia was continuously extracted with hexane for 6 hours, then with acetone for 18 hours. The resulting yellow solution was reduced *in vacuo* to give an orange solid which was partitioned between water and chloroform. The chloroform was dried and reduced *in vacuo* to give an orange solid (84mg.), which was purified by preparative thin layer chromatography (chloroform-methanol (98:2)) . The main band ( $R_f$  0.25) was isolated as an orange solid (38mg.).

### Acetylation of Averufin derived from [2- $^{13}\text{C}$ ]-Malonate

Averufin isolated from *A.parasiticus* fed with ethyl [2- $^{13}\text{C}$ ]-malonate (38mg., 0.10mmoles) was acetylated as described for the hexanoic acid-derived averufin (see above). The product was a yellow crystalline solid, *averufin diacetate*, m.p. 258–262°C.



-  $^1\text{H}$  N.M.R. (60 MHz.,  $\text{CDCl}_3$ ): 1.61 (3H, s, 6'- $\text{CH}_3$ ), 1.7-2.2 (6H, m, 3',4',5'-( $\text{CH}_2$ )<sub>3</sub>), 2.38 (3H, s, - $\text{COCH}_3$ ), 2.47 (3H, s, - $\text{COCH}_3$ ), 5.25 (1H, br m, 1'-CH), 7.28 (1H, d,  $J=2.1\text{Hz.}$ , 7-CH), 7.56 (1H, s, 4-CH), 7.98 (1H, d,  $J=2.1\text{Hz.}$ , 5-CH).

-  $^{13}\text{C}$  N.M.R. (91 MHz.,  $\text{CDCl}_3$ ): 15.53 (C-3'), 20.92 (- $\text{COCH}_3$ ), 27.58 (C-6'), 28.10 (C-2'), 35.52 (C-4'), 67.28 (C-1'), 101.11 (C-5'), 111.66 (C-4), 117.44 (C-2), 118.02 (C-5), 123.16 (C-7), 123.26 (C-7 + C-13), 123.41 (C-12), 134.15 (C-14), 135.61 (C-11), 146.94 (C-1), 151.36 (C-8), 154.33 (C-6), 159.14 (C-3), 167.71 (- $\text{COCH}_3$ ), 168.76 (- $\text{COCH}_3$ ), 179.01 (C-9), 180.92 (C-10) ppm.

- M.S. ( $m/z$  (%)) 452 ( $m^+$ ), 410, 393, 368, 352.

#### Attempted isolation of Sterigmatocystin from *A.versicolor*

*A.versicolor* 5219 was stored in darkness at 4°C under paraffin on slopes of Blakelees malt extract agar. A spore suspension was inoculated into twenty-five 50ml. Erlenmeyer flasks containing 20ml. of low salts medium. This was incubated at 26°C in constant light. Every 24 hours for ten days the contents of two flasks were filtered, and the residue was homogenised with acetone. The resulting slurry was filtered, and the filtrate was partitioned between a methanol-water (90:10) mixture and hexane. The methanol layer was concentrated, and partitioned between chloroform and water. The organic layer was dried and reduced *in vacuo* to give a crude extract used for high performance liquid chromatography analysis.

Each sample was dissolved in 2ml. of equal quantities of analar acetonitrile

and distilled water. After filtration, this was applied to a reverse-phase high performance liquid chromatography column and eluted with distilled water in analar acetonitrile (50:50). One peak had the same retention time as a standard sample of sterigmatocystin. However when eluted with the same solvents in a (30:70) proportion, no peak had the same retention time as this standard sample. This was confirmed by analytical thin layer chromatography; using various solvent systems (acetone-chloroform (10:90), acetone-benzene (5:95) and chloroform-methanol (96:4)) gave  $R_f$  values of 0.7, 0.5 and 0.7 respectively for a standard sample of sterigmatocystin, with a characteristic orange-red spot under ultra-violet irradiation (264nm.). No such spot was observed for any *A.versicolor* crude extract.

#### Growth Production Curve of aflatoxin B<sub>1</sub> in *A.flavus*

*A. flavus* 120920 was stored in darkness at 4°C under paraffin on slopes of Blakelees malt extract agar. A spore suspension was inoculated into twenty-five 250ml. Erlenmeyer flasks containing 100ml. of low salts medium. This was incubated at 26°C in constant light.

The growth media were decanted off the mycelia, which were homogenised in a methanol-water (90:10) mixture. The resulting slurry was filtered under reduced pressure, and the filtrate (50ml.) washed with hexane (50ml.). The filtrate was then concentrated reduced *in vacuo*, and the aqueous residue was partitioned between chloroform and more water (50ml. each). The orange organic solution was dried and reduced *in vacuo*. This repeated over nine days to give a crude extract which was weighed.

The relative amount of aflatoxin B<sub>1</sub> in each crude extract was monitored by reverse-phase high performance liquid chromatography. Samples were dissolved in 10ml. of a (50:50) mixture of acetonitrile and distilled water and

applied to a reverse-phase high performance liquid chromatography column. Analar acetonitrile and distilled water were used as the moving phase, with a variety of programmed gradients which increased the proportion of water in the mixture. For any programme used, a peak corresponding in retention time to that of an authentic sample of aflatoxin B<sub>1</sub>, was observed from extracts of three days' growth or later.

### *[7-<sup>2</sup>H]-Averufin*

Sodium methoxide (20mg) was dissolved in dry [<sup>2</sup>H]-methanol. Averufin (200mg., 0.54mmoles) was added, giving a purple solution; this was stirred under reflux for ten hours. The product was acidified (2N hydrochloric acid), giving an orange precipitate which was taken up in chloroform. This was washed, dried and reduced *in vacuo* to give an orange solid which was purified by preparative thin layer chromatography (chloroform-methanol (96:4)). An orange solid, *[7-<sup>2</sup>H]-averufin* (170mg., 85% returned) by analytical thin layer chromatography, was isolated.

- <sup>1</sup>H N.M.R. (80 MHz., (CD<sub>3</sub>)<sub>2</sub>SO<sub>2</sub>-CDCl<sub>3</sub>): 1.49 (3H, s, 6'-CH<sub>3</sub>), 1.54-2.05 (6H, m, 3',4',5'-(CH<sub>2</sub>)<sub>3</sub>), 5.25 (1H, br m, 1'-CH), 6.52 (d, J=2.4Hz., residual 7-CH), 7.05 (1H, s, 4-CH), 7.11 (1H, d, J=2.1Hz., 5-CH), 12.15 (s, exch, ArOH) ppm.

- M.S. (m/z (%)): 369 (M<sup>+</sup>, 53), 368 (29), 326 (73), 311 (100), 310 (95), 296 (57).

*[4',6'-<sup>2</sup>H<sub>5</sub>]-Averufin* Cooled deuterium oxide (5ml.) was slowly added to phosphorous pentachloride (5g.); a vigorous reaction ensued. The solution was

stirred for 30 minutes then added to a solution of averufin (760mg., 2.06mmoles) in dry tetrahydrofuran (50ml.). The orange solution was stirred under reflux for three days, then extracted into chloroform (500ml.). This was washed, dried and reduced *in vacuo*, to give an orange solid, mainly  $[4',6'-^2H_5]$ -averufin by analytical thin layer chromatography (714mg., 94% returned).

-  $^2H$  N.M.R. (55 MHz.,  $(CH_3)_2SO_2-CHCl_3$ ): 1.71, 2.12 ppm.

- M.S. (m/z (%)): 374 ( $m^+$ , 11), 373 (33), 372 (62), 371 (63), 370 (39), 369 (18), 368 (7), 327 (7), 310 (100), 297 (87), 256 (75).

#### **Incorporation of labelled averufins into aflatoxin B<sub>1</sub> by *A.flavus***

A mixture of 7- $^2H$ ]-averufin and 4',6'- $^2H_5$ ]-averufin (100mg. of each) were dissolved with difficulty in acetone (20ml.). A spore suspension of *A.flavus* 120920 was inoculated into 50ml. Erlenmeyer flasks containing 20ml. of low salts medium. This was incubated at 26°C in constant light. Of this, 5ml. was distributed among 3 flasks 24 hours after inoculation. This was repeated after a further 36, 48 and 60 hours. A further 24 hours later, the flasks' contents were worked up as described previously. A small amount of pale yellow material was isolated; this corresponded to an authentic sample of aflatoxin B<sub>1</sub> by analytical thin layer chromatography.

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## Lecture Courses

Lecture courses attended during the period of research include:

- "Current Topics in Organic Chemistry" (5 lectures each year), 1982-1985, various lecturers.
- "Mycology" (5 lectures), by Dr. J.W. Deacon (Microbiology Department, University of Edinburgh, 1982.
- "The Chemistry of Photographic Processes" (5 lectures) by Dr. L.A. Williams (Kodak), 1983.
- "Pulse Sequences and Applications in N.M.R. Spectroscopy" (5 lectures), by Dr. G.A. Morris (University of Manchester), 1983.
- "1,3-Dipolar Intermediates in Heterocyclic Synthesis" (5 lectures), by Drs. J.T. Sharp and R.M. Paton, 1983.
- "Industrial Chemistry", (6 lectures), by Drs. Nicoll, Mustoe and Sinclair (Paisley College of Technology), 1985.
- Organic Research Seminars (4 years attendance), various speakers.

Publication



## **Studies of Polyketide Chain Assembly Processes: Incorporation of [2-<sup>13</sup>C]Malonate into Averufin in *Aspergillus parasiticus***

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Analysis of the <sup>13</sup>C n.m.r. spectrum of averufin (**1**) enriched by feeding diethyl [2-<sup>13</sup>C]malonate to cultures of *Aspergillus parasiticus* shows high and equal incorporation of <sup>13</sup>C-label at *nine* positions to indicate a clear acetate 'starter' effect; thus averufin is a decaketide, not an octaketide derived from a hexanoate starter and seven malonates as suggested by recent studies.

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## Studies of Polyketide Chain Assembly Processes: Incorporation of [2-<sup>13</sup>C]Malonate into Averufin in *Aspergillus parasiticus*

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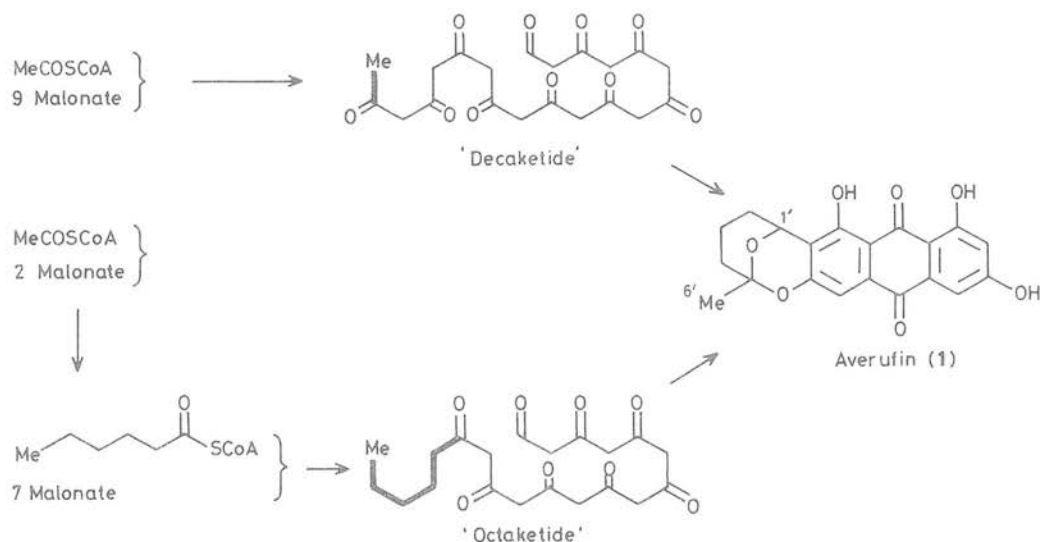
Analysis of the <sup>13</sup>C n.m.r. spectrum of averufin (1) enriched by feeding diethyl [2-<sup>13</sup>C]malonate to cultures of *Aspergillus parasiticus* shows high and equal incorporation of <sup>13</sup>C-label at *nine* positions to indicate a clear acetate 'starter' effect; thus averufin is a decaketide, not an octaketide derived from a hexanoate starter and seven malonates as suggested by recent studies.

The polyketide pathway<sup>1</sup> is one of the major pathways of secondary metabolism but until recently very little was known of the processes involved in the early stages of polyketide biosynthesis. However, recent studies<sup>2</sup> using precursors labelled with the stable isotopes <sup>13</sup>C, <sup>2</sup>H, and <sup>18</sup>O have provided significant indirect evidence for the nature of the enzyme-bound intermediates produced by the polyketide-synthesising enzymes in different organisms, and for the sequence of events involved in their assembly. These studies indicate that reduction and loss of oxygen in polyketide biosynthesis occurs by mechanisms similar to those involved in fatty acid biosynthesis,<sup>3</sup> during and not after the chain elongation process. Although there has been little success in attempts to obtain direct information on the necessary intermediates which appear to be enzyme-bound throughout the assembly process, the intact incorporation of <sup>13</sup>C-labelled hexanoate into averufin was recently reported.<sup>4</sup> Feeding [1-<sup>13</sup>C]hexanoic acid to cultures of *Aspergillus parasiticus* resulted in high specific incorporation of label at C-1' of averufin (1). This was a most significant observation and led to the proposal that averufin, and therefore the aflatoxins, were

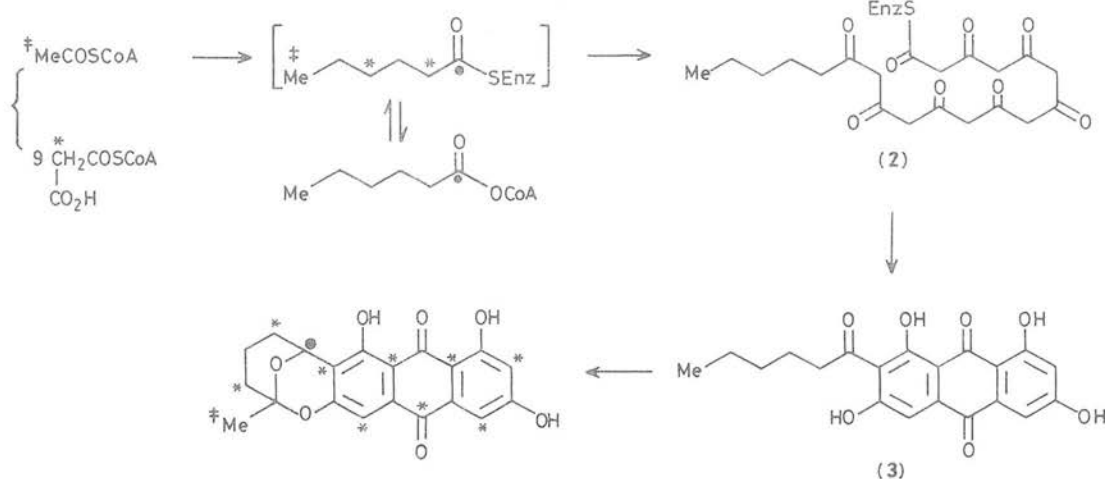
not decaketides as previously accepted,<sup>5</sup> but rather that they were octaketides, being formed by elongation of a separate, previously formed hexanoate starter by addition of seven malonates as indicated in Scheme 1. However, a possible alternative interpretation is that a single decaketide synthetase is involved which produces an enzyme-bound C<sub>6</sub> segment which can exchange with free hexanoyl CoA (Scheme 2). In order to test this hypothesis further we have examined the incorporation of <sup>13</sup>C-labelled malonate into averufin.

Diethyl [2-<sup>13</sup>C]malonate was prepared from sodium [2-<sup>13</sup>C]-acetate<sup>6,7</sup> and fed to static cultures of *A. parasiticus*. <sup>13</sup>C N.m.r. analysis† of the enriched averufin revealed that high

† Averufin was converted into the triacetate for n.m.r. analysis. 50 MHz <sup>13</sup>C n.m.r. spectra were determined in CDCl<sub>3</sub> using inverse gated decoupling conditions to suppress nuclear Overhauser enhancement (n.O.e.) effects<sup>8</sup> in the presence of the paramagnetic relaxation agent trisacetylacetonatochromium.<sup>9</sup> Percentage enrichments<sup>10</sup> were obtained by comparing the intensities of resonances in both the natural abundance and enriched spectra after normalisation.<sup>11</sup>



Scheme 1. Alternative octaketide and decaketide routes to averufin (1).

Scheme 2. Proposed pathway for incorporation of malonate into averufin.  $[2\text{-}^{13}\text{C}]$ malonate enrichment:  $1.1 \pm 0.1\%$  (\*),  $0.4 \pm 0.2\%$  ( $^{\dagger}$ ).

and essentially equal incorporation of  $^{13}\text{C}$ -label from malonate had occurred at *nine* of the possible positions as indicated in Scheme 2, with only C-6' showing a lower level of incorporation. This clearly demonstrates that an acetate starter effect is operating. Thus it appears that averufin is indeed a decaketide being formed from an acetate starter unit by addition of two successive malonates with a full reduction-elimination-reduction sequence, *cf.* fatty acid synthetase, following each condensation step to produce enzyme-bound hexanoate. Assembly then continues by successive condensation of seven further malonates with no further reduction steps to give the requisite precursor (2) for direct cyclisation to produce norsolorinic acid (3)<sup>5</sup> as the first enzyme-free intermediate and subsequently averufin.

The significance of the previous observations,<sup>4</sup> therefore, is that exogenous hexanoate can equilibrate with the enzyme-bound intermediate and so be incorporated intact without prior degradation. This suggests that if intermediates with the correct oxidation level are fed under the right conditions then success can be achieved in the direct study of polyketide chain assembly processes.

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